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Molecular Medicine

RNA Interference: From Basic Research to Therapeutic Applications

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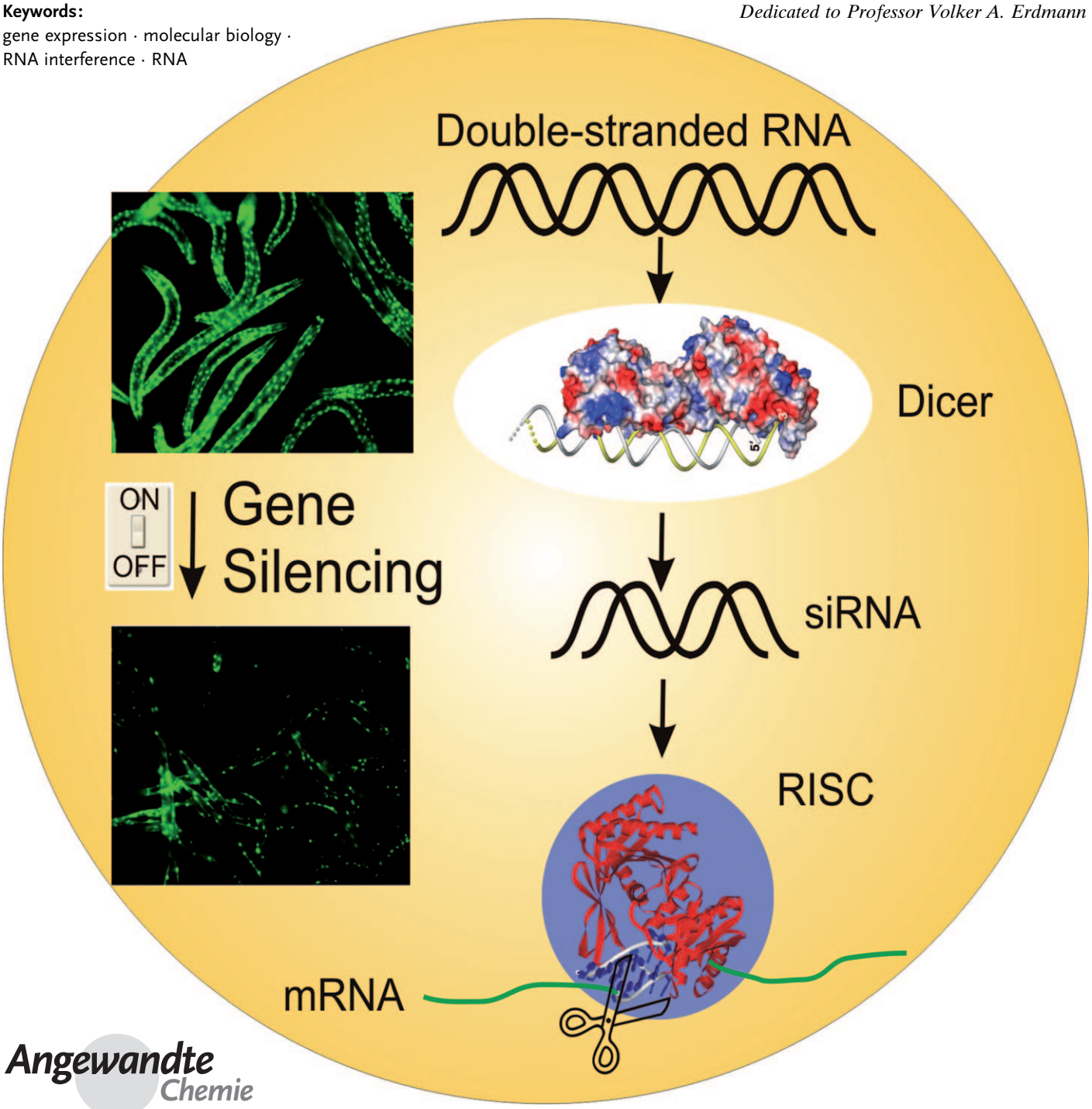
Dedicated to Professor Volker A. Erdmann



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Only ten years ago Andrew Fire and Craig Mello were able to show that double-stranded RNA molecules could inhibit the expression of homologous genes in eukaryotes. This process, termed RNA interference, has developed into a standard method of molecular biology. This Review provides an overview of the molecular processes involved, with a particular focus on the posttranscriptional inhibition of gene expression in mammalian cells, the possible applications in research, and the results of the first clinical studies.

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1. Introduction

The term RNA interference (RNAi) refers to a cellular process by which a double-stranded RNA (dsRNA) sequence-specifically inhibits the expression of a gene. This very efficient process of posttranscriptional gene silencing (PTGS), which involves numerous cellular proteins besides the RNA, is strongly conserved in eukaryotes and presumably serves as a protection against viruses and genetic instability arising from mobile genetic elements such as transposons. It was originally observed in plants,[1] but correctly described for the first time in the late 1990s for the nematode *Caenorhabditis elegans*.[2] For this achievement Andrew Fire and Craig Mello were honored with the 2006 Nobel Prize for Medicine or Physiology.[3,4] As measured by the number of publications, RNAi belongs, along with proteomics, to the most dynamic fields of biotechnology.[5]

1.1. The Mechanism of RNA Interference

In their key publication, Fire and Mello introduced a long double-stranded RNA into *C. elegans* and observed that, as a result, the expression of the homologous gene was blocked.[2] Since then, the basic processes involved have been determined in detail. In a first step, the endonuclease Dicer processes the long dsRNA into small or short interfering RNAs (siRNAs) which are around 21 nucleotides long, of which 19 nucleotides form a helix and 2 nucleotides on each of the 3′ ends are unpaired (Figure 1 A). The actual effector of the RNAi is the ribonucleoprotein complex RISC (RNA-induced silencing complex), which is guided by the siRNA to the complementary target RNA. As a result, the target RNA is cleaved at a specific site in the center of the duplex, 10 nucleotides from the 5′ end of the siRNA strand.[6] The catalytic component that cleaves the target RNA (slicer activity), has been identified as the protein designated Argonaut 2 (Ago2).[7] An analysis of its crystal structure showed that Ago2 contains a domain which resembles RNase H,[8] a long-known protein that cleaves the RNA component of a DNA/RNA duplex. After cleavage, the target RNA lacks those elements which are typically responsible for stabilizing mRNAs, namely the 5′ end cap and the poly-A tail at the 3′ end, so that the cleaved mRNA is rapidly degraded by RNases and the coded protein can no longer be synthesized.

It is assumed that the loading of the siRNA into the RISC is accomplished by the RISC-loading complex (RLC), which consists in *Drosophila melanogaster* of Dicer-2 and R2D2, and in mammalian cells of Dicer and the TAR RNA binding protein (TRBP). Furthermore, it has been shown that during the activation of the RISC, the strand designated the passenger (or sense) strand is cleaved, while the other strand, the guide (or antisense) strand, remains in the RISC.[10,11] Recent investigations with reconstituted human RLC demonstrated that Ago2 dissociates from the rest of the complex after loading with the double-stranded RNA.[12]

1.2. RNA Interference in Mammalian Cells

1.2.1. siRNAs for Targeted Inhibition of Gene Expression

The technique of turning off the expression of specific genes by dsRNAs could, initially, be applied to a large number of eukaryotes, such as plants, *C. elegans* or *D. melanogaster*, but could not be applied to mammals since long dsRNAs trigger an unspecific interferon (INF) response in mammalian cells. The dsRNA is interpreted by these cells as a pathogen, and protein kinase R is activated, which terminates protein synthesis in the affected cells.[13] Furthermore, enzymes are induced which produce 2′-5′-linked oligoadenylates and thereby cause an RNase L-dependent unspecific degradation of single-stranded RNA.[14]

Since the INF response is only triggered by dsRNAs which are longer than 30 nucleotides,[15] the realization that RNAi is induced by RNAs of approximately 21 nucleotides[6,16]

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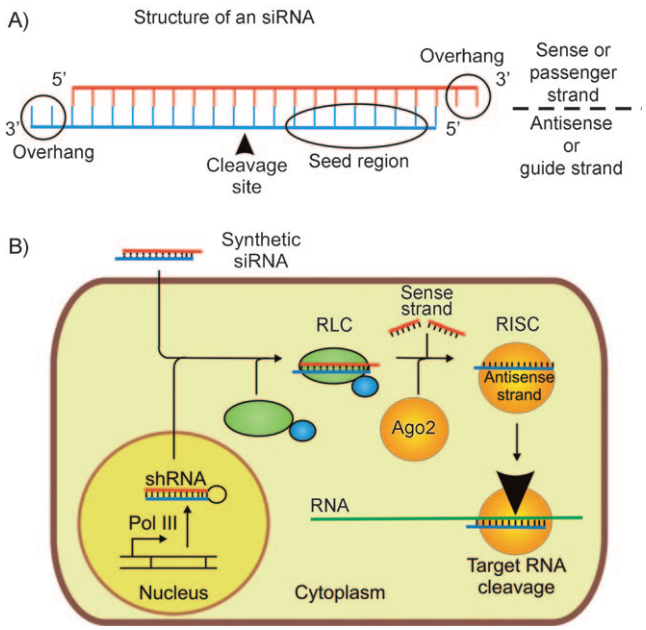

Figure 1. A) Structure of an siRNA. The two strands of the siRNA form an approximately 19 nucleotide duplex. Two nucleotides hang over from each 3′ end. Deoxythymidine is often used as the overhangs in chemically synthesized siRNAs. The position at which the complementary target RNA is cleaved is indicated with an arrow, and the seed region, through which the interaction with the target RNA begins, is indicated. B) Simplified model of the RNAi mechanism in mammalian cells. After uptake of the chemically synthesized siRNAs into the cells, they are loaded onto the RISC by the RLC, in the course of which the sense strand is removed. The antisense strand guides the RISC to the complementary target RNA, which is cleaved by the Ago2 protein. A longer term inhibition of gene expression can be accomplished when an shRNA is expressed intracellularly instead of by the exogenous application of an siRNA. (Figure adapted from Ref. [9].)

provided a solution to the problem: With their groundbreaking work that chemically synthesized 21-mer siRNAs trigger RNAi in mammalian cells, Tuschl and co-workers opened the way to use RNAi for experiments in mammalian cells.[17] This created new opportunities, not only for research, but also for therapeutic treatments. The presynthesized siRNA is phosphorylated on its 5′ end by the kinase Clp1 after entering the cells[18] which is then followed by the RNAi pathway described above (Figure 1B).

RNAi expanded the repertoire of the already well known oligonucleotide-based strategies of PTGS. Antisense oligonucleotides have been employed for the last 30 years to inhibit the expression of genes at the mRNA level. Antisense and RNAi strategies have many things in common, such as the necessity to identify suitable binding sequences on the target RNA, the stabilization of the oligonucleotide by chemical modification, or the transport of the negatively charged polymer across the cell membrane. Experience in the antisense field allowed for very rapid progress to be made with the new RNAi strategy.[19] There are, however, important differences between the two technologies: Antisense oligonucleotides are single-stranded (modified) DNA molecules, which primarily induce the cleavage of the target RNA in the cell nucleus by activation of RNase H. In contrast, RNAi is triggered by double-stranded RNA, which functions primarily in the cytoplasm. Ago2, the most important component of the RISC, is localized in the p bodies.[20] As a result, the central steps of RNAi appear to take place in these discrete structures of the cytoplasm. In the case of RNAi, an endogenous cellular pathway is followed, which could explain the high efficiency with which siRNAs are able to inhibit the expression of their target genes. They can be up to 1000 times as efficient as traditional antisense oligonucleotides against the same target molecule.[21,22] While no particularly important region could be determined for the normally 15–20 nucleotide long antisense oligonucleotides, the seed region (positions 2–8 of the antisense strand, Figure 1A) is of great importance for siRNAs, since it is presumably here that the interaction with the target RNA begins.

The effects of siRNAs are transient. The degradation of the target RNA usually begins immediately after the siRNA enters the cell; however, the decrease in the amount of protein depends on the half-life of the target protein. Normally a pronounced inhibitory effect can be observed in cell culture within 48 h of transfection of an siRNA; however, there are proteins with a very slow rate of turnover, which can be stable for much longer. Also one must keep in mind that in most cases the target gene is not completely shut off, which is why RNAi is referred to as a knockdown technology as opposed to knockout in the case of transgene animals created by homologous recombination.

The inhibition of the expression of the target gene usually lasts for five to seven days both in vitro[23] and in vivo.[24] Interestingly an siRNA can work for different lengths of time in different species: An siRNA against apolipoprotein B was active in mice for only a few days and after nine days was back to 70% of its initial starting level, whereas the knockdown in nonhuman primates was still effective after 11 days.[25] The duration of action of an siRNA presumably depends on numerous factors, such as the target organ, the target gene, and the species. Intracellularly expressed short hairpin RNAs (shRNAs) can be used instead of chemically synthesized siRNA to extend gene silencing (see Figure 1B and Section 3).

RNAi is primarily a process of PTGS, that is, gene expression is inhibited by a selective blockade of an mRNA. It has also been reported that RNAi can alter the chromatin structure in the nucleus and thereby influence transcription.[26] This has been observed in particular for yeast, plants, and fruit flies. The importance of RNAi for transcriptional

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gene silencing in mammals has, in contrast, not yet been clearly demonstrated.

1.2.2. *Endogenous Short RNAs: miRNAs, piRNAs, and esiRNAs*

Besides the previously described siRNAs, which can be employed as research tools and potential therapeutics for the artificial regulation of gene expression, the importance of endogenous short RNAs which do not code for proteins is becoming increasing clear. The role of the approximately 21 nucleotide long micro-RNAs (miRNAs) in the posttranscriptional regulation of genes has been investigated very intensively in the last few years.[27] In Version 11.0 of the miRBase data base (http://microrna.sanger.ac.uk) from April 2008 there are over 6000 miRNAs listed from animals, plants, and viruses; for humans alone over 1000 miRNAs are predicted.

In the nucleus, miRNA precursors (pri-miRNAs) are formed by special miRNA genes or as introns from protein-coding polymerase II transcripts. They are processed by the RNase III Drosha to approximately 70 nucleotide long pre-miRNAs, which are transported out of the nucleus by Exportin-5 and are cleaved there by Dicer to become the functional miRNAs (Figure 2). Similar to siRNAs, they also form a ribonucleoprotein complex with Argonaut proteins and bind to their target RNAs. However miRNAs preferentially recognize target sequences in the 3′-untranslated region (3′-UTR) of mRNAs, and binding often takes place with an incomplete homology, although a perfect base pairing in the previously mentioned seed region (positions 2–8 of the miRNA) forms the core of the interaction. Depending on the degree of homology between the miRNA and mRNA, the result can be an irreversible cleavage of the target molecule or merely repression of translation.

The precise mechanism of the miRNA-dependent post-translational repression of gene expression is currently the subject of intense research.[27] According to the two most important models, either translation is blocked or the mRNA is destabilized. The inhibition of translation could take place at the level of initiation. In this process it is assumed that the Ago2/miRNA complex interacts with the cap structure at the 5′ end after binding to the 3′-UTR of an mRNA and thereby prevents the binding of the initiation factor eIF4E. As a result, the initiation complex cannot be formed. Alternatively, translation could be slowed after initiation or the ribosomes could dissociate prematurely. According to the alternative model, the mRNA is de-adenylated by the miRNA, which makes 3′→5′ degradation possible or the cap is removed, which would enable degradation in the 5′→3′ direction by exonucleases. Possibly there are other mechanisms through which miRNAs could work.

It is assumed that miRNAs control the activity of about 30% of all protein-coding genes in mammals. Since every miRNA regulates numerous mRNAs, and in turn mRNAs can be influenced by numerous miRNAs, this results in an extremely complex regulatory network. So it is hardly surprising that miRNAs are involved in all cellular processes that have been investigated and play an important role in numerous diseases, such as cancer,[28] viral infections,[28,29] and genetic diseases.[30] For further Information concerning the activity and function of miRNAs the reader is referred to recent review articles.[27,31]

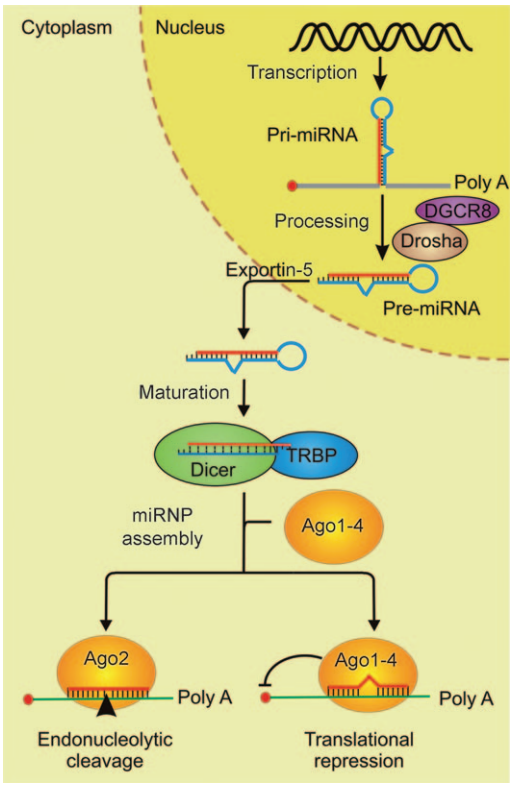

Figure 2. miRNA pathways in mammalian cells. RNAs are transcribed in the nucleus in the form of a precursor (pri-miRNA), which is processed by the RNase III Drosha to pre-miRNA. In this process, the Drosha complexes with the DGCR8 protein. The pre-miRNA is exported out of the nucleus and into the cytoplasm by Exportin-5 and cleaved there by Dicer (complexed with TRBP) to form the functional miRNA, which in turn combines with an Argonaut protein (Ago) to form an miRNA–ribonucleoprotein (miRNP) complex. The miRNA can either cause endonucleolytic cleavage of the target mRNA through Ago2 or block translation in the case of partial complementarity. (Figure adapted from Ref. [27].)

A further class of short regulatory RNAs are associated with Piwi proteins and are thus referred to as piRNAs.[32] These RNAs, at around 24–30 nucleotides in length, are slightly longer than typical siRNAs or miRNAs. They are presumably processed from single-stranded precursors and are found principally in germ cells. Besides their importance in the control of mobile genetic elements, a function in spermatogenesis is also suspected.

Recently, endogenous siRNAs (esiRNAs) were found by comprehensive sequencing of short RNAs in mammalian cells (mouse oocytes).[33,34] It was previously assumed that an RNA-dependent RNA polymerase activity was required for the production of esiRNAs, but this is not found in mammals. It has now been shown that other double-stranded RNAs, such as long hairpin structures or complementary sequences, can serve as the starting point for the production of esiRNAs. The esiRNAs derive from retro-transposons and apparently function as their inhibitors. In addition, esiRNAs from

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pseudogenes have been found, which could be of significance for the regulation of protein-coding transcripts.

2. Design and Stabilization of siRNAs

2.1. Design of siRNAs

The first important step for the successful application of RNAi is the design of efficient siRNAs. The original assumption, that it is not necessary to search for suitable target sequences in the target RNA,[35] proved to be too optimistic. In practice, the efficiency of different siRNAs against the same target RNA varies drastically.[36] Apparently factors intrinsic to the siRNA itself and the characteristics of the target RNA both play a role in silencing.[37]

The probability of identifying a very efficient siRNA was significantly increased after it was discovered that both strands of an siRNA or miRNA are not equally likely to be incorporated into a RISC. Instead, the strand with a lower thermodynamic stability (namely, a higher A/T content) at its 5′ end is preferred.[38,39] Thereafter, the molecular basis for this strand asymmetry could be determined.[40] In *D. melanogaster*, RISC is loaded by a heterodimer of Dicer-2 and the dsRNA-binding protein R2D2. Here, R2D2 binds to the more thermodynamically stable end of the double-stranded RNA and thus determines which strand associates with the RISC as the guide strand. In a detailed study with 180 siRNAs against two different RNA targets, besides the relative stability of the two ends, additional criteria (preference for special bases in certain positions) were identified which are common among the functional siRNAs.[41]

In these experiments, however, the significance of each parameter was determined independently of one another. To also take into account synergistic influences of multiple linked parameters, an artificial neuronal network was trained with a dataset of over 2000 siRNAs against 34 different mRNAs (BIOPREDsi-algorithm).[42]

An extensive survey of the activity of published siRNAs has shown, however, that there are a number of very active siRNAs which do not correspond to the proposed criteria, while numerous other carefully designed siRNAs are inactive. Recently, even the hypothesis that the relative stability of the two ends has an influence on their efficiency has been called into question.[43] Neither in an experimentally investigated set of different siRNAs nor in a comprehensive analysis of published siRNAs or siRNAs posted to databanks could a correlation between the terminal stability of the siRNA and its silencing activity be found. Other characteristics of the siRNA also possibly play a role. For example, it has been shown that siRNAs whose antisense strands form stable helices at their ends only show a low level of activity.[44] The authors advise, therefore, designing siRNAs such that the antisense strand is as unstructured as possible.

Besides the siRNA itself, the target RNA could also play an important role in silencing. This could help to explain why the expression of some targets is easily inhibited, while the knockdown of others is more difficult. In a study with several thousand siRNAs, which were conceived for different genes according to the BIOPREDsi algorithm, 70% of the investigated kinase genes were easy silenced (defined as two of two tested siRNAs working), while 6% of the genes could not be down-regulated by up to 10 different siRNAs.[45]

Studies with antisense oligonucleotides have already shown that the accessibility of the binding region on the target RNA of oligonucleotides is of great importance for the efficiency of silencing. A correspondence between the accessibility for antisense oligonucleotides and siRNAs has been demonstrated.[46] In a more comprehensive analysis, the accessibility of target RNAs was predicted by an iterative bioinformatic approach and by experimental RNase H mapping.[47] The results showed that siRNAs against predicted highly accessible areas were more efficient than those whose target sequence was inaccessible. The relative thermodynamic stability of the two ends of the siRNA proved, in contrast, not to be a suitable criterion for the prediction of the efficiency of an siRNA.

We analyzed the influence on silencing of the thermodynamic design of the siRNA and the accessibility of the target RNA more closely with the help of artificial target structures.[48] We were able to confirm in reporter assays the strand asymmetry, namely, that the target sequences in the natural orientation led to a stronger silencing than the other way around. On the other hand, there was a clear correlation between the local free energy of the siRNA binding region and silencing. We therefore proposed a two-step model to describe the inhibitory efficiency of siRNAs (Figure 3): Initially the thermodynamic characteristics of the siRNA, that is, the relative stability of the two ends, determine the asymmetric incorporation of the two strands into the RISC. In a second step the accessibility of the binding region of the

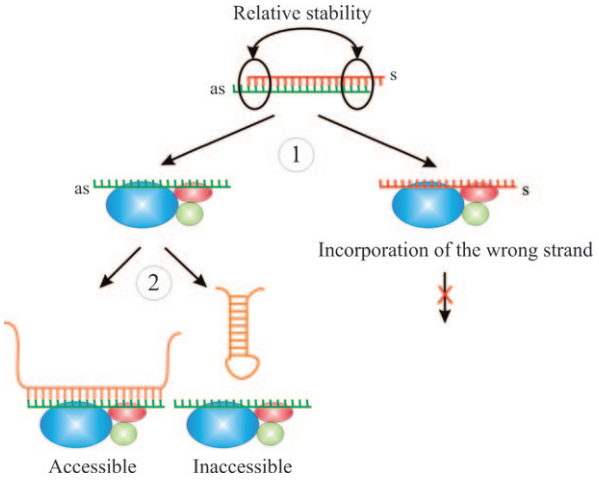

Figure 3. Two-step model to explain the efficiency of siRNA (s: sense strand, as: antisense strand): 1) Depending on the relative stability of the two ends of an siRNA, one of the two strands is preferentially assembled into the RISC. The retention of the strand complementary to the target RNA can be achieved through the selection of a suitable sequence. 2) An antisense strand assembled into the RISC can, however, be unsuitable for silencing when the complementary sequence of the target RNA is inaccessible. The local structure of the target region thus also influences silencing significantly. (Figure adapted from Ref. [48] with permission from Elsevier.)

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siRNA on the target RNA affects the strength of the silencing. This model was confirmed in an analysis of around 200 siRNAs and shRNAs against over 100 different human genes.[49] According to this study, the accessibility of the target RNA for the siRNA is of greater importance than the duplex asymmetry for efficient knockdown. In a further report it was shown that the accessibility of the 3′ end of the target RNA is particularly important.[50] As already mentioned in Section 1, the interaction between the siRNA or miRNA and the target RNA begins in the seed region.

Some algorithms for the design of siRNAs, such as the Sfold web server,[51] take into account not only the thermodynamic characteristics of the duplex but also the predicted secondary structure of the target RNA. It must be emphasized that none of the models proposed so far can guarantee a successful prediction of the activity of an siRNA. There must, therefore, be other factors which still need to be identified, in particular synergistic effects, which influence the efficiency of RNAi experiments.

Conventional siRNAs consist of a 19-mer duplex and two nucleotide long overhangs on each of the 3′ ends. It has, however, been reported that longer siRNAs can be more efficient. In an experiment with siRNAs of various lengths, 27 mers had an efficiency up to 100 times higher than the conventional 21 mers.[52] In a further study, 29-mer shRNAs were proven to be especially potent.[53] The long duplexes were initially processed to 21 mers by Dicer and were thus presumably more efficiently assembled into the RISC by the RLC.

The problem of the design of individual siRNAs can be bypassed by the use of enzymatically synthesized siRNA pools.[54] First, long dsRNAs are generated which can be processed with bacterially synthesized RNase III or recombinant Dicer to endoribonuclease-prepared siRNAs. This mix of siRNAs can harbor the risk of increased off-target effects (see Section 4); on the other hand, each individual siRNA is present at a very low concentration so that the undesirable side effects are apparently diluted out. With this method, inexpensive comprehensive libraries against the complete human and mouse genome have been manufactured.

2.2. Chemical Modification of siRNAs

Although unmodified siRNAs can be used in cell cultures, it can be advantageous to build modified nucleotides into the siRNA so as to specifically inhibit the expression of a gene. The primary reason for the chemical modification of siRNAs is the increase in resistance to nucleolytic degradation. In fact, although siRNAs have an unexpectedly long life, it is usually necessary to stabilize them further by the use of modified nucleotides for in vivo applications. Modifications can often lengthen the half-life of the siRNA in plasma and improve its pharmacokinetic characteristics. Furthermore, new functionalities can be introduced, fluorescent markers or lipophilic groups, for example, which improve cellular uptake. The rapid successful incorporation of chemically modified components can be attributed to the experience gained in the field of antisense technologies. A multitude of modified nucleotides have been employed in the past years for siRNAs, of which several selected examples will be explained here. Further details have been explained extensively in comprehensive review articles.[55–57]

The incorporation of unnatural nucleotides into siRNAs presents a particular challenge, since the modifications must not affect the silencing activity of the siRNA. In this context it is important to remember that the two strands of the siRNA have different functions: while the guide strand is assembled into the RISC and leads the complex to the target RNA, the passenger strand is discarded in loading the RISC. The passenger strand is, therefore, more likely to tolerate modifications, but the guide strand can also have modified nucleotides built into suitable positions.

Of particular importance is the hydroxy group at the 5′ end of the guide strand, which must be phosphorylated for entry of the siRNA into the RNAi pathway. Correspondingly, an siRNA whose 5′ end is blocked—for example, by an amino linker—loses its inhibitory activity.[58] Comparatively simple, in contrast, is the incorporation of functional groups on the ends of the passenger strand. In this way it is possible to follow the localization of an siRNA with a fluorophore on the 5′ end of the passenger strand without having a grave influence on its silencing activity.[59] Furthermore, the cellular penetration of the siRNA can be improved with a lipophilic component such as 12-hydroxylauryl acid or cholesterol (see also Section 5.1.1).[60]

The most common modification for the stabilization of antisense oligonucleotides is phosphorothioate DNA, in which an unlinked oxygen atom is substituted by a sulfur atom. Phosphorothioates are very stable with respect to nucleases and are comparatively simple to manufacture. RNA variants of the phosphorothioates (Figure 4) have therefore also been built into siRNAs. These modifications are fundamentally tolerated by the RNAi machinery; however, toxic side effects have been observed when the phosphorothioate content is high.[61]

Nucleotides have also been used whose ribose was modified at the 2′-position, for example, 2′-*O*-methyl RNA

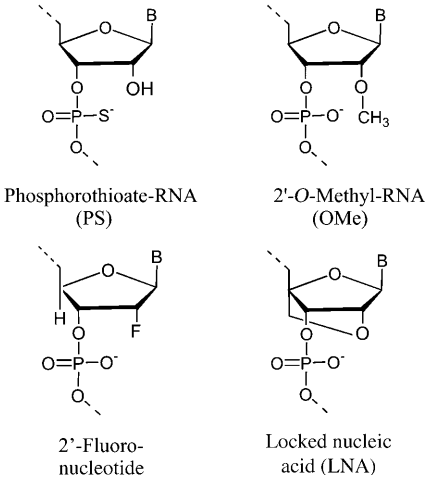

Figure 4. Selected modified nucleotides which can be employed to stabilize siRNAs.

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and 2′-fluoro-modified nucleotides (Figure 4). The fluoro substituent is very small, and does not seriously influence the functionality of the siRNA.[61–63] The significantly larger methyl group, in contrast, inhibits the RNAi function when the entire siRNA consists of 2′-*O*-methyl-substituted nucleotides.[63] Therefore, modification types are sought which increase the stability of siRNA without reducing their silencing activity. Blunt-ended siRNAs proved to be suitable when the RNA units and 2′-*O*-methyl nucleotides alternate in both strands, so that a modified nucleotide is opposite an unmodified one.[64] Such modified siRNAs were injected into mice as components of lipoplexes (see Section 5.1.1).[65] The siRNAs were taken up by vascular endothelial cells and reduced the level of the target mRNA and of the target protein.

A further modification commonly used in past years is the locked nucleic acid (LNA, Figure 4).[66–68] LNAs have numerous desirable characteristics such as high nuclease stability and high affinity for the target structure; their incorporation into an RNA duplex, however, causes serious structural changes. A complete modification of an siRNA with LNAs is therefore impossible, but a few LNA monomers can be built into the siRNA without loss of its silencing ability.[62] In a systematic study, the positions of the antisense strand were identified which tolerate the substitution of the RNA nucleotides by an LNA component without loss of activity.[69] The incorporation of LNAs into siRNAs not only increases the nuclease stability, it can also reduce the off-target effects of an siRNA (see Section 4) by inactivating the sense strand and increase the efficiency of siRNAs by improved loading of the RISC. Corresponding LNA-modified siRNAs showed favorable characteristics in systemic use in vivo compared to unmodified siRNAs.[70]

We used the method of inactivating a strand of an siRNA by the incorporation of LNAs to analyze in detail the mechanism of RNAi-induced inhibition of the coxsackie virus.[71] These cardiotropic viruses, which belong to the family of the *Picornaviridae*, possess a single positive-stranded genome, from which during replication a negative strand is copied as an intermediate. The selective inactivation of one of the two strands by LNAs showed that only siRNAs against the genomic positive strand possess an antiviral activity.

In a further study, a triple-stranded siRNA construct was employed, in which the antisense strand was hybridized with two shorter 10–12 nucleotide long complementary strands.[72] These so-called small internally segmented interfering RNAs (sisiRNA) were modified at various positions with LNAs and had a very high serum stability and silencing activity.

The fact that the various modifications in different positions of the siRNA could be built into the siRNA without a drastic loss of activity suggested the possibility of combining various types of RNA analogues. In this way all of the OH groups of an siRNA could be substituted successfully: all the pyrimidines were replaced by 2′-fluoro-modified nucleotides, the purines of the sense strand by deoxyribonucleotides, while 2′-*O*-methyl-RNAs were used for the purines of the antisense strand.[73] Furthermore, the ends were protected by inverted abasic sugars and a phosphorothioate bond. These completely modified siRNAs had a half-life in human serum of several days, as opposed to several minutes for their unmodified forms, and were significantly more efficient than the starting siRNA in a vector-based in vivo model of hepatitis B virus (HBV) infection.

3. Vector Expression of shRNAs

A great disadvantage of chemically synthesized siRNAs is that their activity is transient and only lasts several days, because the siRNAs degrade over time and are diluted out by cell division. It was, therefore, a major advance when in 2002 several research groups simultaneously developed expression systems in which the siRNA is continuously generated in cells.[74]

3.1. Expression Plasmids for shRNAs

In the most common system the siRNA is converted into a DNA sequence which codes for the sense strand, a loop, and the antisense strand. This DNA template is transcribed from a vector under the control of polymerase III promoters. These promoters are optimized for the generation of large amounts of precisely defined RNAs. The most commonly used are the promoter of the U6 component of the spliceosome as well as the H1 promoter of the RNA component of RNase P. During transcription, a self-complementary RNA is created, which is referred to as an shRNA. The shRNA is processed intracellularly by Dicer into siRNA, which mediates silencing.

The shRNA expression systems led to the creation of new applications for RNAi. Usually a vector-expressed shRNA works significantly longer than chemically synthesized siRNA. Plasmids equipped with a resistance gene can be used to select transfected cell lines in which the target gene can be down-regulated for several months.[75]

In addition, transgenic animals can be generated in which the gene of interest is permanently inhibited by using shRNA expression vectors. For example, the shRNA expression cassette can be incorporated into embryonic mouse stem cells by electroporation[76] or lentiviral transfer[77] (see Section 5.2.1). A problem of this method is that integration of the transgene is random, so the silencing efficiency can vary considerably depending on the integration site. Furthermore, important cellular genes can be destroyed. For this reason a locus was sought that guaranteed a strong and predictable shRNA expression. The *Rosa 26* locus fulfils these requirements and is used to integrate the transgene homologously by recombinase-mediate cassette exchange (RMCE). The knockdown was 80–95 % when there was a single copy of the shRNA-expression cassette in most analyzed organs.[78] An advantage of this procedure relative to conventional knockout techniques is the immense saving in time: The shRNA-expressing animals are available for investigation in around three to four months, while with knockout animals back-crosses that can take up to several years are often necessary before the gene can be deleted from both chromosomes in a genetically defined background.

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Surprisingly, we have recently observed phenotypic differences between knockout and shRNA-expressing animals.[79] In this study, the function of the vanilloid receptor TRPV1, which plays an important role in pain perception, was investigated in detail. While the reaction of the shRNA-expressing animals was in accordance with published data from TRPV1-knockout animals in most tests, such as capsaicin-induced hypothermia and colitis, and their reaction to a heat stimulus, they showed pronounced differences in the perception of neuropathic pain. While the knockout of TRPV1 had no impact on the perception of neuropathic pain, the mechanical hypersensitivity and allodynia in the shRNA-expressing animals was significantly reduced in comparison to wild-type animals. This finding agrees with results from small molecule receptor antagonists.[80] The cause for the differences in the behavior of knockout and shRNA-expressing animals is not yet fully understood; however, a complete knockout and a partial knockdown appear to lead to differences in compensation mechanisms. One should keep in mind that small molecule pharmacological substances also only partially inhibit their targets, so that the partial knockdown in an RNAi experiment may better reflect the outcome of a medicinal therapy with substances directed against that target.

A further advantage of the RNAi technology is its broad applicability. While classical knockouts by homologous recombination are only routinely done with mice, shRNA vector technology allows genes to be turned off in other species, such as rats.[81] A further development of this idea is the creation of disease-resistant domestic animals with the help of RNAi. In goat foetuses and bovine blastocysts, RNAi shut off the prion protein (PrP), which aggregates in transmissible spongiform encephalopathy (TSE).[82] In this way it was possible to generate domestic animals which are resistant to BSE and related diseases. Cattle could be made resistant to foot-and-mouth disease by using a similar method. The creation of transgenic domestic animals, however, not only results in technological challenges, but also has ethical and social implications which must not be neglected.

3.2. MicroRNA-Type shRNAs

While the shRNAs in the systems described so far are expressed under the control of polymerase III promoters, modern systems can also employ polymerase II promoters. This results in transcripts with a cap at the 5′ end and a poly-A tail at the 3′ end, which are not compatible with the RNAi machinery. Nevertheless, to use polymerase II promoters, the expression of miRNAs is simulated. These alternatives are usually components of longer pre-mRNAs which are transcribed under the control of polymerase II promoters. A naturally occurring miRNA can be replaced with an artificial shRNA in the sequence context of the miRNA.[83] The RNA polymerase II first generates a long primary transcript, from which Drosha cuts out the pre-miRNAs. These are exported into the cytoplasm where Dicer processes them into siRNAs, which are assembled into RISC.

Comparative studies with conventional and miRNA-type shRNAs against HIV have shown that the latter are up to 80% more efficient.[84] Besides their high efficiency, the miRNA-type shRNAs have other advantages relative to classical shRNAs. For one thing, they allow the simultaneous expression of a protein-coding sequence upstream of the miRNA segment. In this way, a reporter such as GFP or a relevant functional protein can be expressed with the shRNA at the same time. Secondly, polycistronic expression becomes possible, that is, more than one microRNA-type shRNA can be expressed at the same time from a single transcript.[85] In this manner, either several genes can be silenced at once or a target gene can be very efficiently inhibited by several shRNAs. A third advantage is the option of using cell-type-specific promoters. While polymerase III promoters mediate a strong and ubiquitous expression, there are a large number of different polymerase II promoters which are only active under certain conditions or in specific cell types. For example, an miRNA-type shRNA against the transcription factor Wilm's Tumor 1 was expressed under the control of the proximal promoter of the murine gene *Rhox5*. This specifically inhibited the expression of the target gene in nurse cells of the testis.[86]

3.3. Inducible Systems

The vector systems also provided the opportunity to regulate RNAi by pharmacological substances. These systems may be differentiated into reversible and irreversible types. In reversible systems, expression of the shRNA is "turned on" by the addition of an inducer. When the inducer is taken away, transcription of the shRNA ceases and the target gene of the siRNA is once again expressed. In irreversible systems, shRNA expression can be induced, but cannot be turned off again. This form of regulation is widely employed when genes which are essential for embryonic development are to be investigated in adult organisms.

The most common reversible shRNA expression system is based on tetracycline (tet) controlled transcription.[87] For tet control, the promoter is usually modified by the addition of a tet operon, to which a repressor protein binds. The addition of an inducer—such as tetracycline or its more commonly used structural analogue doxycycline (dox)—results in a structural change in the tet repressor being induced such that it is released from the tet operon, which opens the way for transcription of the shRNA.

The tet system functions in vitro as well as in vivo. For example, an shRNA against the polo-like kinase 1 (Plk1) was dox-dependently expressed to study the importance of the target gene for the proliferation of cancer cells.[88] It was shown by inoculating these cells into immunodeficient nude mice that the RNAi-mediated silencing could be modulated in a dox-dependent manner in vivo. In a further study, transgenic animals were generated according to the previously described RMCE procedure, in which the shRNA expression could be reversibly induced by dox.[89] In this way, the target gene, which codes for the insulin receptor, could be down-regulated for a chosen period of time.

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The tet system combines numerous advantages: It has a low background activity in the absence of an inducer, is strongly inducible, and quickly reversible after removal of the inducer, and the inducers tetracycline and doxycycline are nontoxic, well-characterized pharmacological substances. Besides the described system, there are numerous other variants of tet control and other reversible regulation systems, which are explained in a recent review article.[90]

The cre-lox system has been widely used for many years for conventional knockouts and has also been employed as an irreversible method for conditional RNAi. In this system, transcription of the functional shRNA is destroyed by the insertion of an additional DNA segment into the expression cassette. For example, a neomycin (neo) resistance gene flanked by two *loxP* sites can be integrated into the shRNA-coding region.[91] CRE recombinase removes the interrupting sequence when expressed in the same cell and induces the synthesis of the shRNA. Alternatively, the *stuffer* sequence can also be inserted in the promoter region. Cre-lox systems allow temporal control of RNAi suppression, for example, induction after embryonic development as well as tissue-specific silencing when CRE recombinase is expressed in certain cell types.

4. Unspecific Side Effects

4.1. Off-Target Effects

Small molecular pharmacological substances which typically bind to proteins and inhibit their catalytic cores or block membrane-bound receptors usually bind to their target molecules through spatial interactions. This often results in undesirable side effects when the substance also binds to other structurally similar proteins. Since RNAi applications are based on Watson–Crick base pairing between an oligonucleotide and an RNA, there was hope that undesired side effects played no role when a target sequence that only appears once in the genome was used. In practice, a single mismatch can lead to a complete loss of silencing.[75,92]

More extensive microarray analyses, with which global profiles of gene expression can be created, showed, however, that siRNAs are not completely specific. While initial studies suggested that the so-called off-target effects of siRNAs are dose-dependent and can be avoided by the use of lower concentrations of siRNA,[93] other studies showed that the unspecific effects have a similar dose response to the intended knockdown of the target gene.[94] The identity of as few as eleven nucleotides between the antisense strand of the siRNA and an mRNA can result in the down-regulation of an mRNA which is not the intended target. These off-target effects can have effects on the phenotype, for example, the viability of cells.[95]

More recent investigations have shown that it is not the overall identity of an mRNA with the siRNA, but rather the perfect correspondence between parts of the 3′-UTR and the seed region (positions 2–7 or 2-8) of the antisense strand of the siRNA which determines whether gene expression is influenced (Figure 5).[96] In a systematic study the frequency

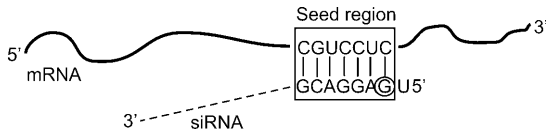

Figure 5. Base pairing between nucleotides 2–8 of the siRNA (seed region) and mRNAs can lead to off-target effects in RNAi applications. These undesired side effects can be significantly reduced by a 2′-*O*-methyl substitution of the second nucleotide (circled).

of all 4096 possible hexamers in the 3′-UTR of the transcriptome was investigated.[97] It was shown that some hexamers are rare while others are considerably more common. It became clear in a microarray analysis that siRNAs with common seed regions trigger stronger off-target effects than those for which there are only a few complementary sequences. This means that off-target effects can be reduced by clever design of the siRNA. Furthermore, the specificity of siRNAs can be reduced through the incorporation of modified nucleotides. It is comparatively easy to completely inactivate the sense strand by modifications so that the danger of off-target regulation can be reduced to a minimum. Changes to the antisense strand are, on the other hand, more challenging since the inhibitory effects on the expression of the target gene must not be influenced. A single 2′-*O*-methyl substitution on the ribose of the second nucleotide was shown to be enough to significantly reduce off-target effects while maintaining silencing activity (Figure 5).[98]

4.2. Interferon (INF) Response

Besides the regulation of partially homologous mRNAs, siRNAs can surprisingly also trigger an interferon (INF) response, although it was originally assumed that these responses are only induced by double-stranded RNA molecules greater than 30 nucleotides in length. A complete analysis of the INF-stimulated genes revealed, however, that siRNAs can also activate the interferon system, presumably mediated by protein kinase R.[99] This effect is not specific for siRNAs, but has also been observed for vector-expressed shRNAs.[100]

Presumably the Toll-like receptors (TLR) and the helicases RIG-1 and Mda5, in addition to protein kinase R, also play an important role in the recognition of siRNAs by the immune system. Three members of the TLR family recognize RNA and can trigger an immune response through a complex signaling pathway (Figure 6). It could be shown for plasmacytoid dendritic cells that siRNAs induce INF-α via TLR7.[101] The activating effects of the siRNAs on endosomal TLRs is dependent on the sequence of the siRNA.[102] As a result, motifs could be identified which led to a strong induction of the immune response. This means that immunostimulation can be circumvented by avoiding the use of these motives in an siRNA. For special applications, such as the treatment of viral infections or cancer, strongly immunomodulatory siRNAs which have two functions, knockdown of the target

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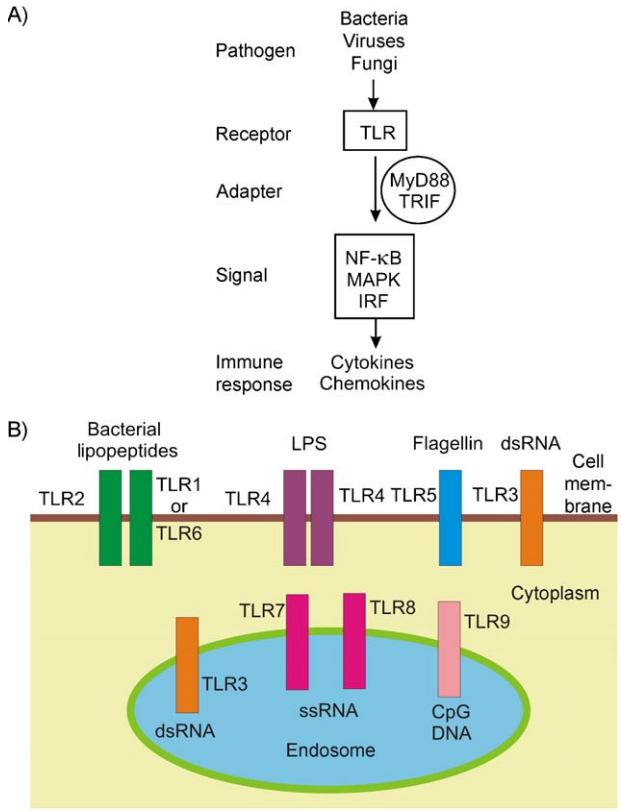

Figure 6. Toll-like Receptors (TLR). A) Signaling pathway of the TLRs. B) Cellular localization and ligands which activate the various TLRs. LPS: Lipopolysaccharide; CpG: cytidine-phosphate-guanosine; MyD88: myeloid differentiation primary response protein 88; NF-κB: nuclear factor kappa beta; MAPK: mitogen-activated protein kinases; IRF: interferon regulatory factor. (Figure modified from Ref. [104].)

gene and induction of interferons, could be used deliberately.[103]

In a recent publication it was reported that unspecific effects of siRNAs can also be mediated by TLR3. The investigation of the anti-angiogenetic effects of siRNAs, which are used, for example, in the treatment of age-related macular degeneration (see Section 6.3.1), showed in an animal model that unspecific siRNAs without homologous sequences in the mammalian genome were just as efficient as siRNA against the vascular endothelial growth factor (VEGF) or its receptor.[105] These effects were not dependent on a sequence-specific silencing of the target, nor were off-target RNAi nor INF-α/β activated. Instead, choroidal neovascularization was blocked by TLR-3 and its adaptor TRIF, which are localized in various cell types of the cell surface, as well as the induction of INF-γ and interleukin-12.

4.3. Cross-Reactions with the miRNA Pathway

Further undesirable side effects can come about by cross-reactions with the endogenous miRNA pathway. As explained in the Introduction, siRNAs and miRNAs function by very similar mechanisms. For this reason it is hardly surprising that siRNAs can act as miRNAs. This means that siRNAs can interact with the 3′-UTR of mRNAs by partial homology and can inhibit their translation without triggering their degradation.[106,107]

Furthermore, expressed shRNAs can block the endogenous miRNA pathway. A pronounced liver toxicity was observed after a high dose of viral vectors carrying an shRNA expression cassette was injected into mice.[108] Of the 49 shRNAs tested, 36 caused liver damage, which in 23 cases was fatal. Presumably, the cellular miRNA pathway was disturbed by, among other things, over-saturation of Exportin-5, which is responsible for transporting miRNA precursors out of the nucleus and into the cytoplasm. No side effects were observed at a lower concentration of the vector, in contrast, and protection from HBV was achieved in an animal model for up to a year. In response to this work, a recently published study investigated whether chemically synthesized siRNAs have an influence on cellular miRNAs.[109] Liposomal delivery of the siRNAs resulted in the expression of hepatocyte-specific genes being inhibited by around 80%. The level and the function of several investigated miRNAs were not influenced by the siRNA treatment.

In conclusion, it is clear that RNAi applications will never be completely specific. By suitable design of the siRNAs as well as the use of modified nucleotides, however, the unspecific effects can be minimized. In addition, the dose of the siRNAs or shRNAs should be as low as possible. The reliability of the results of functional analyses can be increased by verifying the phenotype with multiple independent siRNAs. For therapeutic applications, it must be remembered that small molecular substances usually also have numerous (toxic) side effects. For this reason, the same safety standards should apply to the preclinical development of RNAi applications as for other substances.

5. Delivery

Oligonucleotides are multiply negatively charged macromolecules which cross the hydrophobic cell membrane with difficulty. The delivery of the siRNAs into cells presents one of the greatest challenges to the development of RNAi applications. For cell-culture applications, transfection reagents are commonly used, which often have toxic side effects in animals or humans. Previous work from the antisense field established that a certain amount of oligonucleotides are spontaneously taken up by cells in vivo. Thus, siRNAs also work without a carrier when locally applied, such as through intranasal delivery[110] or intrathecal injection.[24] It should be remembered in this case that local application can create a high concentration of the siRNA in a spatially restricted area. Additional measures are required for efficient systemic delivery. Basically, the approaches can be divided into nonviral delivery of chemically synthesized siRNAs and viral delivery of shRNA expression cassettes. The preferred method depends on the application: for temporary diseases such as acute infections, the short-acting siRNAs can be sufficient, while for chronic diseases, such as HIV infection or metabolic diseases, the vector-based method is presumably more advantageous to avoid repeated dosing.

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5.1. Nonviral Delivery

In the first in vivo applications of RNAi free siRNAs were applied by hydrodynamic injection in to the tail vein.[111] This involves injection of a relatively large volume of the siRNA solution in a short time at high pressure. In this way the siRNAs are preferentially taken up by the liver, and proof of principle was demonstrated in practice by the knockdown of target genes in this target organ. This method is, however, very harsh and not viable for humans. For this reason, intensive work on the development of biocompatible procedures for the delivery of siRNAs has been going on for many years.

In vivo systemic application of siRNAs requires that they overcome numerous barriers to unfold their activity:[112] Free oligonucleotides are rapidly filtered from the blood by the kidneys and subsequently excreted. In addition, unmodified siRNAs are rapidly degraded by nucleases (see Section 2.2), and foreign macromolecules are phagocytized by the reticuloendothelial system and deposited in the liver and spleen. The half-life of the siRNAs in the bloodstream can be extended by hydrophobic polymers such as polyethylene glycol (PEG). The siRNAs must then overcome the capillary endothelium and diffuse into the extracellular matrix of the target tissue. Uptake into the cells normally occurs by endocytosis, during which an important step is the release of the siRNA from the endosomes into the cytoplasm, where they manifest their activity. There are numerous methods to aid these processes, of which the most important will be discussed here.

5.1.1. Unspecific Delivery

Many substances are packed into liposomes to improve their pharmacokinetic characteristics. Liposomes form a phospholipid bilayer surrounding an aqueous compartment, in which polar substances can be stored, and mediate uptake of the substances into the cells by some form of vesicular transport, for example, through endosomes. Cationic lipids are particularly well suited for the delivery of negatively charged nucleic acids. Most commercially available transfection reagents form lipoplexes with the oligonucleotides; in these lipoplexes the siRNA is not contained in the inner compartment. However, numerous new, less-toxic formulations have been developed for in vivo applications. Usually lipoplexes and liposomes are surrounded by PEG (Figure 7A,B) to achieve longer circulation in the blood stream and to reduce the toxicity. In addition, fusogenic lipids can be added, which improve the release of the siRNAs from the endosomes. While free siRNAs are rapidly excreted by the kidneys after intravenous injection, an siRNA labeled with the fluorescent dye Cy3 that was administered as an siRNA lipoplex could be detected in many organs.[65] The siRNAs remained, unfortunately, primarily in the endothelial cells of the blood vessels and, therefore, barely penetrated into the tissues themselves.

Liposomal delivery of the completely modified siRNA against HBV described in Section 2.2 increased both the efficiency and duration of action in a mouse model.[113] The siRNA was encapsulated in stable nucleic acid-lipid particles (SNALPs), which consist of a cationic and a fusogenic lipid and are also PEGylated (Figure 7B). SNALPs were subsequently used to test an siRNA against apolipoprotein B in primates.[25] The level of the mRNA in the liver was reduced by more than 90% after a single injection, and as a result the protein, the serum cholesterol, and the level of low-density lipoprotein (LDL) was reduced. This showed that liposome-mediated siRNA delivery could be successfully tested in a clinically relevant context. The knockdown of apolipoprotein B demonstrates two further aspects: First, a partial reduction of the target protein is sufficient to reach a relevant therapeutic benefit, namely reduction of LDL to a normal level. For the use of RNAi against tumors or viral infections, however, the greatest possible knockdown of the target gene must be reached to prevent a relapse of the disease. Secondly,

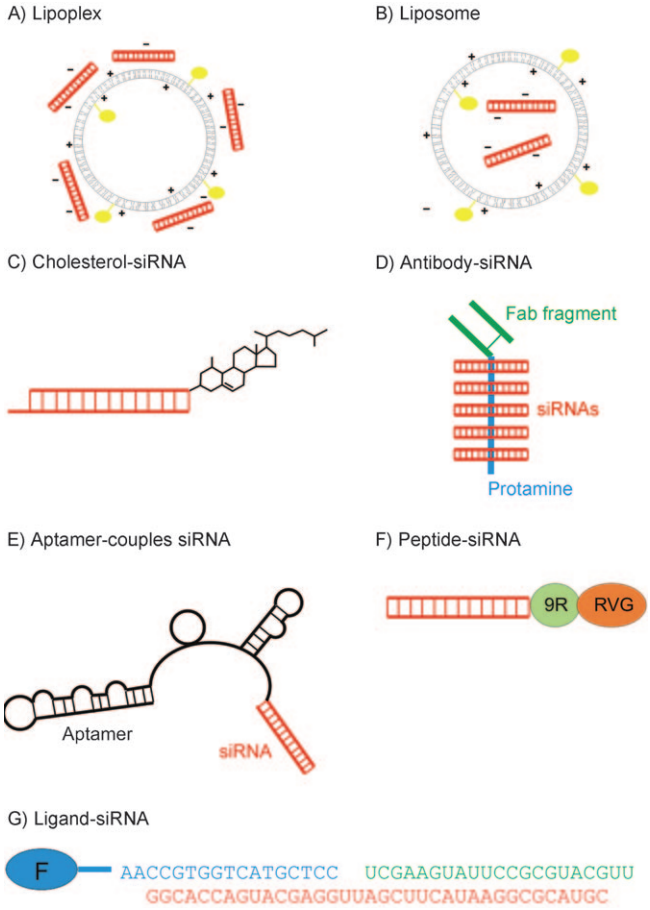

Figure 7. Nonviral delivery of siRNAs. A) Lipoplex: cationic lipids (gray) form complexes with the negatively charged siRNAs (red). PEG (yellow) is frequently attached to improve the pharmacokinetic characteristics. B) Liposomes in which the cationic lipids enclose the siRNA. C) siRNA coupled to cholesterol to increase its lipophilicity. D) Specific delivery by coupling of siRNAs on the antigen-binding fragment of an antibody through positively charged protamine. E) Direct coupling of an siRNA to an aptamer for tumor-cell-specific delivery. F) Neuronal delivery by a peptide of the rabies virus glycoprotein (RVG) with an arginine nonamer (9R) at its carboxy terminus to bind the siRNA. G) Receptor-mediated delivery by coupling of a ligand (F: Folate) to a DNA oligonucleotide (blue), that hybridizes with siRNA (sense strand: green, antisense strand: red). Further details are described in the text.

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the advantages of RNAi technology lies in the fact that any chosen gene can be inhibited, not just the so-called drugable targets, against which traditional small-molecule substances can be directed.

Besides the lipid-based systems, various other polymers have been employed for the delivery of siRNAs. One of the most intensively investigated polymers for the delivery of nucleic acids is polyethyleneimine (PEI). The linear or branched PEI polymers are strongly positively charged and can therefore form complexes with siRNAs and electrostatically interact with the cell surface. The complexes are taken up by endocytosis, and PEI improves the release of the siRNA by destroying the endosomes. PEI-siRNA complexes can be employed successfully to limit influenza infections in mice, for example.[114]

Nanoparticles from completely different substances have also been developed. For example, Medarova et al. used nanoparticles, which after systemic application allow the delivery and proof of siRNA uptake at the same time.[115] The samples consisted of magnetic nanoparticles labeled with a dye which absorbs in the near-infrared region so that accumulation in tumors could be observed by magnetic resonance imaging (MRI) and near-infrared in vivo optical imaging (NIRF). The nanoparticles were equipped with a myristoyl-coupled polyarginine peptide for translocation across the membrane. In an alternative system, carbon fiber nanotubes were used, which facilitated entry of siRNAs into T cells and primary cells, which are otherwise difficult to transfect with liposomal systems.[116]

An alternative strategy is to couple lipophilic molecules directly to the siRNA. Of a series of tested groups, cholesterol and 12-hydroxylauric acid coupled to the 3′ end of the sense strand proved to be the best suited to ensure efficient uptake by the cells and knockdown of the target gene.[60] As a result, a cholersterol-coupled siRNA (Figure 7C) was injected into the tail veins of mice.[117] Cellular uptake and silencing of the target protein (apolipoprotein B) could be shown in the liver and the jejunum (a section of the small intestine).

In addition to lipophilic groups, cell-penetrating peptides (CPP) can improve the cellular uptake of oligonucleotides.[118] Interestingly, phosphorothioate oligonucleotides which are not covalently attached to an siRNA improve the uptake by a caveolin-mediated mechanism.[119] This resulted in the expression of lamin in primary HUVEC endothelial cells being inhibited.

5.1.2. Cell-Type-Specific Delivery

The development of systems that allow specific delivery of siRNAs to their target cells represents a great advance. This approach allows the applied doses to be smaller and possible side effects in other tissues can be avoided. An elegant possibility consists of coupling the siRNA to an antibody which recognizes a protein on the surface of special cells. In a ground-breaking study, the antigen-binding fragment of an antibody against the HIV glycoprotein, which was fused to protamine, was used (Figure 7D).[120] This positively charged protein can assemble with approximately six (negatively charged) siRNAs in a noncovalent manner. With this construct it was possible to inhibit an HIV infection of primary T cells, which are difficult to transfect with lipid-based strategies. The authors succeeded in vivo with their antibody strategy in delivering the siRNAs to tumor cells which presented the ligand proteins of the antibodies on their cell surface.

To avoid the need to combine two classes of molecules (proteins and nucleic acids) siRNAs have been coupled to aptamers—ligand-binding, in vitro selected nucleic acids. In a first effort, a streptavadin bridge was used to bind an siRNA against lamin to an aptamer against the prostate-specific membrane antigen (PSMA),[121] a membrane receptor which is expressed in prostate cancer cells and the vascular endothelia of tumors. This conjugate made efficient silencing possible, but is relatively complex because of the biotin–streptavadin bridge. For this reason, the siRNA was coupled directly to a different aptamer against PSMA in a further study (Figure 7E).[122] Once in the cell, the siRNA is removed from the aptamer by Dicer. In an animal model it was possible to inhibit the growth of a tumor from human prostate carcinoma cells with an aptamer-coupled siRNA against Plk1.

The treatment of neurological diseases is complicated by the need to pass through the blood–brain barrier, which often prevents the entry of drugs from the bloodstream into the brain. To overcome this barrier, a 29 amino acid long peptide from the rabies virus glycoprotein was coupled with an arginine nonamer to an siRNA (Figure 7F).[123] The peptide bound to the acetylcholine receptor, which is expressed by neuronal cells, so that the conjugate specifically penetrates neurons. In vivo, an intravenously injected siRNA with the peptide succeeded in getting into brain cells, and protected mice from an infection with Japanese encephalitis virus.

A further possibility for cell-type-specific delivery is the coupling of a receptor ligand (such as folate) to a DNA oligonucleotide (Figure 7G).[124] This DNA oligonucleotide hybridizes with the 3′ extended end of the antisense strand of the siRNA, and the ligand mediates the uptake into the cells of the construct, which consists of two RNA molecules and a DNA oligonucleotide. Presumably, Dicer or an RNase H then produces the mature siRNA.

Further details concerning nonviral delivery of siRNAs are described in recently published reviews.[112,125–127]

5.2. Viral Delivery

Viruses belong to the most dangerous pathogens for humans, and therapies against them are often inadequate or not available. For the last 20 years, however, a concept has been pursued to introduce therapeutically useful genes into patients with the help of viral vectors. In the process, the viruses are usually changed such that essential components for replication are missing so that they cannot produce progeny viruses and therefore cannot harm the patient or others (Figure 8). Although worldwide over 220 genes have been transferred in almost 1500 clinical trials,[128] the real breakthrough in gene therapy has not yet been accomplished. High hopes were placed on combining the new and very efficient RNAi technology with the experience of gene

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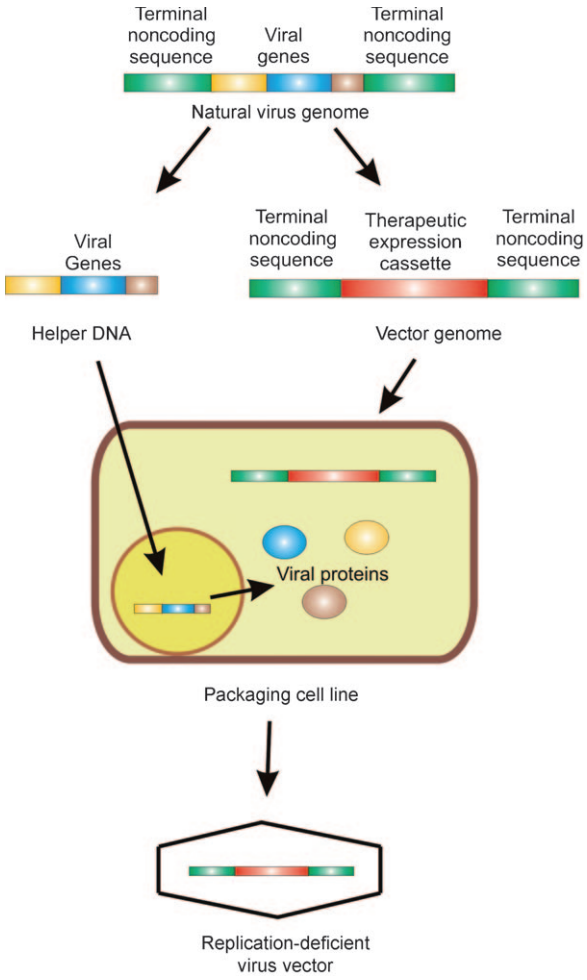

Figure 8. Creation of replication-deficient viral vectors. For gene transfer, the central protein-coding genes of the viral genome are removed and replaced with the transgene or an shRNA expression cassette. The vector genome is packaged in a packaging cell line expressing the viral genes, which in most cases are spread over several plasmids. The resulting virus vector only includes the expression cassette for the transgene, while the essential virus genes are missing, so that further replication is impossible.

therapy.[90,129] This involves getting the shRNA expression cassettes into the cells by means of viral vectors. This form of gene transfer is usually significantly more efficient than the nonviral delivery of siRNAs. Three types of vectors are primarily used: Retroviral vectors, adenoviral vectors, as well as vectors based on the adeno-associated virus (AAV). The most important advantages and disadvantages of the three vector types are summarized in Table 1. Past experience has shown that it is impossible to find a vector which is optimal for all indications, instead the choice of vector type depends on the intended specific therapeutic use.

5.2.1. ***Retroviral Vectors***

Retroviruses have an RNA genome, which is copied into a double-stranded DNA, which in turn is integrated into the host genome as a proviral DNA. This characteristic is maintained in therapeutically used retroviral vectors so as to permanently express the transgene. The immune response to these vectors is weak and the viruses are modified such that they can no longer leave the cells and cause damage.

The family of the *Retroviridae* are divided into the subgroups onco-retroviruses and lentiviruses. Onco-retroviruses can only transduce proliferating cells. They are primarily used ex vivo, that is, the cells—for example hematopoietic stem cells—are removed from the patient which are transduced with the retrovirus vector in tissue-culture dishes and are later re-administered to the patient. In this manner children have been treated who suffer from X-SCID (severe combined immunodeficiency disorder), which is caused by a mutation in the γc interleukin receptor gene located on the X chromosome.[130] The presumed advantage of long-term expression by stable integration into the host genome proved to be a disadvantage, however, since several of the children developed leukemia as a consequence of the treatment.[131] The retroviral vector integrated in the proximity of the LMO2 proto-oncogene promoter and led to the anomalous transcription and expression of LMO2. This finding shows that the safety of the vectors must be improved; at the same time, however, one must remember that diseases such as SCID are frequently untreatable by any other means and lead to the early death of the affected children.

Lentiviral vectors can, for example, be derived from the human immunodeficiency virus (HIV). They have the ability to transduce quiescent as well as proliferating cells, thus increasing their therapeutic range. Furthermore, their oncogenic potential is presumably less. The G glycoprotein of the vesicular stomatitis virus can be used as the coat protein for lentiviral vectors, which allows the transduction of almost any cell type.

After the demonstration that retorviral vectors are in principle suited to siRNA-mediated gene silencing by inhibiting the reporter gene eGFP,[132] they were employed for medically relevant purposes. The specific knockdown of the oncogene *K-Ras*^{V12} allele in human tumor cells caused them to lose their tumorgenicity.[133] In addition, lentiviral vectors were used to introduce shRNA expression cassettes against viruses or their receptors into host cells. A lentivirus vector proved to be particularly efficient for the inhibition of hepatitis C virus (HCV). This vector produced several shRNAs against the virus genome and the host cell receptor

Table 1: Overview of the most important characteristics of viral vectors.

	Retroviral vectors	Adenoviral vectors	Adeno-associated virus vectors
transduction of quiescent cells	no—onco-retrovirus yes—lentivirus	yes	yes
genomic integration	yes	no	no (or limited)
potential risks	insertional mutagenesis	immune reaction, cytotoxicity	cytotoxicity

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CD81 at the same time and thereby blocked HCV replication, CD81 expression, and cell binding of the HCV surface protein E2.[134] The intensive efforts to use lentiviral vectors for the transfer of shRNA expression cassettes for the treatment of HIV infection and an ongoing clinical trial for this purpose will be described in Section 6.3.2.

5.2.2. Adenoviral Vectors

Adenoviruses have a linear, double-stranded DNA genome and most often cause respiratory problems in humans. The genomic DNA of adenoviruses remains episomal in the infected cells, so that no risk of insertional mutagenesis exists. Unfortunately, they do cause a powerful immune reaction, which led to a fatal reaction in a clinical study.[135] Parts of the early genes were removed in the first generation of adenoviral vectors. The early genes *E2* and/or *E4* were deleted in the second generation adenovirus vectors to further reduce the immunogenicity and to create additional space for transgenes. In the newest vectors, which are referred to as gutless, all coding sequences are deleted, so that besides the transgene, only the inverted terminal repeats (ITRs) and the packaging signal Ψ remain.[136] This approach drastically reduces the toxicity and immunogenicity of the vectors, and enables the long-term expression of a transgene.

Adenoviral vectors have already been employed in different medical areas for the knockdown of damaging genes. For example, both guinea pigs and pigs were protected from infection by foot-and-mouth disease by an shRNA-expressing adenovirus vector.[137] The adenovirus vector mediated delivery of shRNA expression cassettes has also been developed for the treatment of heart diseases. The disruption of the calcium balance is an important cause of heart failure. With RNAi-mediated inhibition of phospholamban, an inhibitor of the SERCA2A (sarcoplasmic reticulum Ca^{2+} pump), it was possible to improve the calcium uptake into the sacroplasmic reticulum in primary neonatal rat cardiomyocytes.[138]

5.2.3. Vectors Based on Adeno-Associated Virus

Adeno-associated viruses (AAVs) belong to the family of the *Parvoviridae* and possesses a comparatively small linear single-stranded DNA genome. AAVs are attractive vectors for gene transfer, since they efficiently transduce target cells and are nonpathogenic for humans. While natural AAVs integrate into a specific region in chromosome 19, the genes required for this are usually deleted from the recombinant vectors, so that they remain primarily episomal. Despite this, AAV vectors are noteworthy for their long-term, stable expression of transgenes.

For gene therapeutic uses, the AAV serotype 2 was first developed as a vector. Since it inefficiently transduced many cell types such as muscle cells, other serotypes have been used in the past few years to expand their tropism. The genome of the AAV-2 vector can be packed in capsids of other serotypes. This leads to the creation of so-called pseudotype vectors, with which cells of practically any given tissue can be transduced.[139] A further disadvantage of the conventional single-stranded vectors—the delayed start of the expression of the transgene—could also be eliminated. Maximal gene expression is achieved after only a few days with self-complementary double-stranded AAV vectors.[140]

AAV vectors are intensively employed in RNAi experiments because of their numerous advantages. For example, the dopamine-synthesizing enzyme tyrosine hydroxylase was down-regulated in the midbrain neurons of mice with shRNA-expressing AAV vectors in one of the first in vivo studies.[141] As a result, behavioral changes such as a motor-performance deficit and altered reaction to a psychostimulant were seen. The faster acting self-complementary AAV vectors proved useful for cell-culture experiments: the mRNA of the target gene was reduced by up to 80% after transduction of a culture of rat-lung fibroblasts for 72 h.[142]

6. Applications of RNA Interference

6.1. Investigation of Gene Function

The sequencing of the human genome as well as those of many other eukaryotic model organisms rates as one of the most important developments of the last few decades in the life sciences. In many cases, however, only the sequence is known, while the function of the coded protein remains unknown. Determination of gene function has become one of the most important tasks of present research. At about the same time as the completion of the major sequencing projects, RNAi was established as a method to allow the creation of loss-of-function phenotypes in a comparatively rapid and simple manner. This led to the adoption in only a few years of RNAi as a standard method of molecular biological research that is employed in a very large number of biochemical laboratories.

Since silencing is based on the pairing between the mRNA of the gene of interest and the siRNA guide strand, gene functions can be investigated significantly faster than they can be with small-molecule inhibitors, which must first be identified in laborious high-throughput screens. In addition, closely related isoforms of proteins can be selectively turned off by suitable selection of target sequences to investigate their specific functions,[143] which is almost never possible with pharmacological substances. Even when the goal of a pharmaceutical project is the development of a traditional drug, RNAi offers a rapid method to validate the target.[144]

The unspecific effects of RNAi applications discussed in Section 4 must, however, be kept in mind; thus controls were already laid down with which the specificity of an RNAi experiment should be proven in the early stages of the research.[145] These include, among other things, suitable controls of the knockdown at the mRNA and protein levels, dose–response curves of the siRNA, as well as the use of multiple siRNAs against the same target.

6.2. Screens with Genome-Wide Libraries

Besides the analysis of the function of individual genes, many genes can also be investigated at the same time by using

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siRNA libraries. In a first example, every member of the family of de-ubiquitinating enzymes was selectively turned off with shRNAs.[146] This approach led to the discovery that the knockdown of familial cylindromatosis tumor suppressor (CYLD) led to an increase in the activity of the transcription factor NF-κB after TNF-α stimulation (Figure 9). Interestingly, the activation could be prevented by an aspirin derivative. As a result, patients with cylindromatosis, mostly benign tumors of the skin appendages, were treated with salicylic acid, which in some cases led to a full remission.[147] This example illustrates how RNAi has led to new indications for well-known drugs.

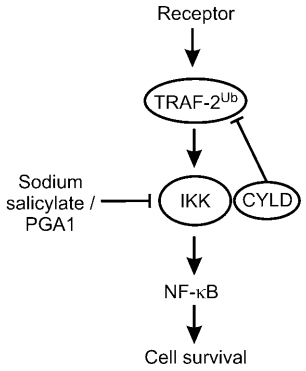

Figure 9. Function of the tumor suppressor CYLD, which was identified by means of an RNAi screen. CYLD works as an inhibitor in the NF-κB signaling pathway. The loss of the CYLD function leads to uncontrolled growth. The pathway can also be inhibited by using sodium salicylate or prostaglandin-1 (PGA1). TRAF: TNF-receptor-associated factor; IKK: IκB kinase complex. Scheme adapted from Ref. [146].

A number of libraries of siRNAs, endoribonuclease-prepared siRNAs, and shRNA-expressing retrovirus vectors have now been developed which cover the entire human genome. Genome-wide screens are primarily used in virological or oncological studies. In this way over 250 cellular factors necessary for HIV-1 infection could be identified in a comprehensive screen with 4 siRNAs against each of the approximately 21000 human genes.[148] This led not only to additional information about the viral life cycle but also identified new potential therapeutic targets. In a screen of retroviral vectors with miRNA-type shRNAs against around 3000 genes, proteins were identified that are involved in the proliferation of cancer cells.[149] In a further genome-wide screen, potential tumor suppressors were found which were required to block the proliferation of fibroblasts and melanocytes that contained an activated mutant of the *braf* proto-oncogene.[150]

6.3. Therapeutic Applications

The decades-long experience with the clinical development of antisense oligonucleotides[151] and ribozymes[152] was utilized in the therapeutic application of siRNAs. Only this can explain how the first RNAi treatments were started on humans just three and a half years after siRNAs were first used in mammalian cells. Antisense oligonucleotides and siRNAs differ from conventional substances by their size, and large-scale synthesis of these oligomers causes considerable difficulties and high costs. Furthermore, the two strands of the siRNAs must be synthesized separately and subsequently hybridized. This process has to guarantee the formation of a uniform drug that must, in the end, satisfy the requirements of the regulatory authorities. Local application was selected for the first proof-of-concept studies because of the previously discussed delivery problems. Table 2 shows the most advanced, RNAi-based clinical trails.

6.3.1. Eye Diseases

The eye is a spatially well-defined organ with low nuclease activity in which the active agent can be injected intravitreally (directly into the vitreous body) comparatively easily. The only two oligonucleotides which have been approved by the American Food and Drug Administration (FDA) are for the treatment of eye diseases. The antisense oligonucleotide Fomivirsen is directed against cytomegalovirus, which causes retinitis in AIDS patients; Macugen is an aptamer for the treatment of age-related macular degeneration (AMD), one of the most common eye diseases among the elderly. The first RNAi-based clinical studies were started at the end of 2004 with an siRNA against VEGF. Inhibiting the expression of VEGF should block neovascularization in patients with AMD. The siRNA has since been tested under the name Bevasiranib in a phase III trial by the company Opko Health.

Sirna Therapeutics (since bought by Merck & Co. Inc., USA) initiated the first clinical studies with a chemically modified siRNA. The siRNA with the code Sirna-027 was stabilized by unpaired deoxythymidine with a phosphorothioate bond and inverted abasic sugar residues on the ends of the antisense and sense strand, respectively, and is directed against the VEGF receptor-1. This approach also enabled the treatment of patients with AMD. An intravitreal injection of the siRNA reduced the area of neovascularization by as much

Table 2: RNAi in clinical trials (based on Ref. [153]).

Company	Disease	Product	Status
Sirna/MERCK	AMD	Sirna-027/AGN211745	phase II
Quark Pharma. (Pfizer)	AMD	RTP801i-14	phase I/II
Opko Health	AMD	Bevasiranib	phase III
Benitec/City of Hope	HIV/AIDS	Lentivirus vector	phase I
Nucleonics Inc.	HBV	NUC B1000	phase I
Alnylam Pharma.	RSV	ALN-RSV-02	phase II
Senetek PLC	glioblastoma multiforme	ATN-RNA	phase I
Calando Pharma.	solid tumors	CALAA-01	phase I
Quark Pharma.	acute renal failure	AKli-5	phase I
TransDerm Inc.	pachyonychia congenita	TD101	phase Ib
Santaris Pharma.	HCV	SPC3649	phase I

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as 66% in a mouse model for choroidal neovascularization.[154] The study of Kleinmann et al.[105] already discussed in Section 4 called the mechanism of action of the siRNAs against VEGF or its receptor into question. The authors came to the conclusion that the antiangiogenic effect was not due to the knockdown of the target genes, but was based on the extracellular activation of TLR-3.

In a further clinical study, the siRNA RTP801i-14 against the hypoxia-induced gene rtp801 was used for the treatment of AMD according to Quark Pharmaceuticals. This approach is possibly safer and more efficient than the anti-VEGF substances.

6.3.2. *Viral Infections*

Viral infections present an increasingly pressing medical problem. The number of chronic infections associated with HIV-1, as well as HBV and HCV, are continually increasing. Furthermore, new variants of viruses, such as the influenza virus H5N1, or new viruses, such as SARS coronavirus, emerge as additional threats. Intensive global travel and the fact that humans and animals live closely together in some regions of the world mean that new dangers from viruses must be expected. Despite the enormous need for antiviral agents, there are only relatively few approved drugs for the treatment of viral diseases. This demonstrates the necessity for the development of new antiviral strategies.

RNAi is based on the complementary base pairing of a target RNA and the guide strand of the siRNA which, as a result, allows for the rapid adaptation of this technology to any given variant of a virus or to new types of virus. This is a great advantage of RNAi relative to conventional approaches, which require time-consuming optimization of small-molecule substances. Since the first reports about the antiviral effects of siRNAs against respiratory syncytial virus (RSV),[155] other successful RNAi applications against most classes of medically relevant viruses, including HIV-1, HBV, HCV, SARS-coronavirus, influenza virus, polio virus, and coxsackie virus, have been published.[156]

An important role in RNAi approaches against viruses is played by the choice of suitable target sequences. Viral RNAs often contain significant secondary structure, which can seriously impede the efficiency of inhibition by an siRNA (see Section 2.1). For example, HIV-1 TAR RNA is inaccessible for the RISC and could only be cleaved after the secondary structure was broken open by 2′-*O*-methyl-RNA oligonucleotides directed against regions neighboring the siRNA binding site.[157]

One of the biggest problems for the long-term use of RNAi against viruses is viral escape. For both the polio virus[158] and HIV,[159] cases have been described in which viral replication can at the beginning be blocked efficiently, but after a while the virus titer increases again, because of the selection of mutants which can overcome the inhibition. Non-essential genes—for example, the *nef* gene of HIV-1—can be deleted.[160] Usually, however, viruses overcome RNAi-mediated silencing with point mutations in the target sequence. A comprehensive analysis of 500 HIV-1 mutants showed that certain positions are preferentially mutated.[161]

To avoid viral escape, siRNAs should be directed against strongly conserved regions of the virus. Nonstructural proteins will be more severely affected by mutations than capsid proteins. It has, however, been reported that substitutions often result in silent mutations which do not affect protein function.[162] This escape route of the virus can be hindered by selecting conserved regions with a structural function which is destroyed by the mutations. In this way, the coxsackie virus could be inhibited over a long period by an siRNA against the conserved cis-acting replication element (CRE), while an siRNA targeted against structurally unimportant regions led to viral escape.[163]

Even with the careful selection of target sequences, however, RNAi approaches will require the development of combination therapy, similar to those already employed in the conventional treatment of viral infections. In analogy to highly active antiretroviral therapy (HAART), in which several small-molecule active drugs are used against HIV-1, several siRNAs or shRNAs could be used against the virus. The combination of four shRNA expression cassettes in a lentiviral vector led to the viral escape of HIV-1 observed for a single shRNA being avoided.[164]

The alternative to the use of siRNAs against the virus is to down-regulate cellular factors which the virus requires to enter the cell and to replicate. The chance of viral escape by mutation is drastically reduced with cellular genes. The critical factor, however, is that the corresponding protein is not essential for the cell. This is, for example, the case for the HIV-1 co-receptor CCR5. Mutations in the *ccr5* gene have no consequences for the health of the individual but protect the person from infection with HIV-1. Hematopoietic stem cells were protected against HIV-1 by the RNAi-mediated knockdown of CCR5.[165] This approach is not, however, restricted to HIV-1—silencing of the coxsackie virus adenovirus receptor led to a significant reduction in the replication of CVB-3.[166–168]

A recently begun clinical trial for the treatment of HIV-1-infected patients combined several targets and RNA-based strategies to get the best protection against escape mutants: A single lentiviral vector expresses an shRNA against the HIV-1 genes *rev* and *tat*, a hammerhead ribozyme against CCR5, and a decoy oligonucleotide against the transactivation response (TAR) element.[169,170] The gene transfer occurs in this case ex vivo, that is, haematopoetic stem cells are removed from the patient, transduced in tissue culture with the vector, and then re-infused.

In a therapeutic program for the treatment of HBV infections, the company Nucleonics Inc. is developing a vector with four shRNAs against different segments of the viral genome. This approach should prevent viral escape. A phase I clinical study with vectors designated NUC B1000 started in 2007.

The lung is one of the organs in which siRNAs are relatively easy to apply; RNAi approaches are thus promising for the treatment of respiratory diseases. Infections with RSV could be inhibited by intranasal application of siRNAs in a mouse model.[110] As a result, a clinical study was initiated to test how well the siRNA ALN-RSV01 were tolerated in healthy volunteers. According to the recently published

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results, no serious side effects were observed, and the systemic bioavailability of the intranasally applied siRNA was minimal, as expected.[171] The subsequent phase II study investigated the safety and antiviral effects of ALN-RSV01 in infected adults. The siRNA was, according to Alnylam Pharmaceuticals, tolerated well and showed statistically significant antiviral activity.

6.3.3. Cancer

A further field in which great hope is placed on RNAi is cancer research.[172] It does not require a great deal of imagination to expect that the inhibition of factors such as oncogenes could block the uncontrolled proliferation of tumor cells. The expression of genes which lead to angiogenesis within the tumor to create new blood vessels to supply the tumor with oxygen and nutrients can also be blocked. In addition, targets may be chosen which are responsible for metastasis, since in most cases primary tumors can be surgically removed and the metastases represent the real problem. Finally, RNAi can be employed to resensitize resistant tumor cells to treatment with chemotherapeutic agents or radiotherapy. The most important way in which tumor cells become resistant to chemotherapeutic agents is through the expression of the multidrug resistance (*mdr*) gene. If MDR expression is suppressed by siRNAs, the cells again become vulnerable to chemotherapeutics.[173]

There are many published studies which show that tumor growth could be slowed in animal models by RNAi. For example, siRNAs against CD31 inhibit the growth of tumors in various xenograft mouse models.[174] The siRNAs penetrate into the tumor endothelial cells as lipoplexes and block angiogenesis.

A further interesting option involves increasing the antitumor activity of oncolytic viruses by RNAi. While viral vectors are usually modified such that after their creation they can no longer replicate (see Section 5.2), oncolytic adenoviruses replicate selectively in cancer cells and destroy the cells by cell lysis. When such a virus is augmented with an shRNA expression cassette (for example, against the mutated $K\text{-}ras^{V12}$ oncogene) the inhibitory effect on tumor growth is increased.[175]

In a first clinical RNAi cancer trial, patients with *Glioblastoma multiforme* were treated.[176] These brain tumors are almost untreatable by currently available means and the prognosis for the affected patients is very poor. The RNAi approach was directed against Tenascin-C, which is strongly expressed in this tumor tissue. The RNAi treatment was successful in preventing the re-emergence of operatively removed glioblastomas in many patients. This product is currently being developed further by Senetek PLC. Calando Pharma employed an unmodified siRNA against the M2 subunit of the ribonucleotide reductase in a phase I study for the treatment of solid tumors, whereby the siRNA was delivered by a special nanoparticle. The company Silence Therapeutics is planning a clinical study with an siRNA lipoplex (Atu027). The liposomal formulated 2′-*O*-methyl-modified siRNA (AtuPLEX) is directed against the expression of protein kinase N3 (PKN3). Other companies have announced clinical trials of RNAi approaches against various forms of cancer for the near future.[177]

6.3.4. Further Clinical Trials

In a further clinical study, RNAi is being employed as a therapeutic strategy against acute kidney failure. It has been shown that the temporary inhibition of the tumor suppressor p53 can prevent cell damage.[178] This will be exploited since the siRNA AKli-5 will inhibit the expression of p53 for a limited period of time. The safety of AKli-5 is to be tested in a phase I trial in patients for whom a high risk of kidney failure exists because of a major cardiovascular operation.

In January 2008, TransDerm Inc. began a clinical study for the treatment of the autosomal-dominant genetic disease Pachyonychia congenita, a disruption of keratinization. The siRNA was intradermally injected and specifically inhibited the expression of the keratin mutation K6a, which is responsible for the disease.[179]

In addition, the blockade of an endogenous miRNA is being tested as a therapeutic strategy. In experiments with nonhuman primates, the liver-specific miRNA-122 could be inhibited by a complementary LNA oligonucleotide.[180] The LNA was systemically (intravenously) administered and did not trigger any apparent toxic side effects. The level of plasma cholesterol could be reduced by inhibiting miRNA-122. This miRNA is an interesting target molecule for a further indication, since it is also required by HCV for replication. According to a press release from the Danish company Santaris Pharma, a clinical trial with the LNA inhibitor of the miRNA-122 began in May 2008.

6.4. Commercial Aspects of RNAi

Since RNAi is a technology which is strongly application-oriented, it is of great commercial significance. The practical application of RNAi rests on several fundamental patents, the most important of which include patents known as Tuschl I and II as well as Kreutzer-Limmer I. While the Tuschl II patent, which refers to the typical 19–21 base pair long siRNAs with 3′ overhangs, has already been granted, the decision regarding the Tuschl I patent remains open. The Kreutzer-Limmer I patent has been granted in Europe (but not yet in the US), however its precise extent is not yet decided.[153] A strong patent position is held by the US Biotech Alnylam Pharmaceuticals. Besides the named core patents on RNAi, they also hold patents on the chemical modification and delivery of the siRNAs. As a result, Alnylam has made several major deals, for example, an extensive research cooperation with Novartis in 2005. In July 2007, Roche AG received from Alnylam a non-exclusive licence for $331 million for the therapeutic use of siRNAs under Alnylam IP and their European research unit. Much attention was generated by the take over of Sirna Therapeutics by Merck & Co., USA, for $1.1 billion at the end of 2006. These transactions show that the major pharmaceutical companies have recognized the potential of RNAi and are prepared to invest a great deal in this new technology. Further details

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regarding the patent situation as well as related business in the RNAi field have been brought together in a recent review.[153]

7. Summary and Outlook

RNA interference has developed into one of the most important technologies of biomedical research within just a few years. The simple and efficient possibility to inhibit the expression of a specific gene makes possible the elucidation of the functions of proteins which are so far unknown. However, RNAi has not only become a standard method of molecular biology—it has already made its way into the clinic. Around a dozen clinical studies based on RNAi are already running, and the first results are promising. Basically, knockdown technologies can be used against any disease in which a deleterious gene is over-expressed (for example, cancer, viral infections, inflammation). Two major obstacles must, however, be overcome before it can become a broadly applicable standard therapy: The question of their specificity and efficient delivery to the target cells. As already explained, siRNAs can cause unspecific side effects and activate the immune system. These undesired effects can be minimized by the clever selection of the sequence and the use of modified nucleotides.

Immense efforts have been undertaken to develop carrier systems with which siRNAs can be delivered to their target cells. Despite the advances of the last years, further developments are still required to get systemically applied siRNAs to their required site of action. Here, viral vector systems for shRNA expression cassettes offer additional options for efficient and organ-specific delivery. This approach must, however, first overcome the reservations based on the negative experience with gene therapy. Then the two strategies—the delivery of chemically synthesized siRNAs and the vector-mediated expression of shRNAs—can complement each other, and either of the approaches can be chosen depending on the requirements of a given application.

With the anticipated advances in the next few years in solving these problems, the vision of many RNAi researchers could become reality: The use of genome-wide screens with siRNA libraries will allow targets for diseases such as cancer to be identified, which then can be functionally investigated and validated with the siRNA employed in the screening. Afterwards, the same molecule can be optimized with chemical modifications in a standard manner and tested in animal models with special delivery agents, before the siRNA (or a corresponding shRNA) can be employed directly for testing in humans. This approach will enable an unprecedented acceleration of the development of new therapy options to be achieved.

With siRNAs, the specific inhibition of a single target gene is usually attempted; however, experience in the antisense field has shown that this can, under some circumstances, be inadequate for complex diseases such as cancer. In contrast, miRNAs affect many target RNAs, so that more comprehensive regulation can be achieved with the inhibition of a miRNA. The clinical studies on the inhibition of miRNAs, which have already begun or are planned for the near future, will possibly show the greater therapeutic effect. The coming years will show whether RNAi, after its success in the research laboratories, will also live up to the promise of the antisense strategies to offer a new medical option for a molecular-based therapy.

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[1] C. Napoli, C. Lemieux, R. Jorgensen, *Plant Cell* **1990**, *2*, 279–289.
[2] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, C. C. Mello, *Nature* **1998**, *391*, 806–811.
[3] C. C. Mello, *Angew. Chem.* **2007**, *119*, 7114–7124; *Angew. Chem. Int. Ed.* **2007**, *46*, 6985–6994.
[4] A. Z. Fire, *Angew. Chem.* **2007**, *119*, 7094–7113; *Angew. Chem. Int. Ed.* **2007**, *46*, 6966–6984.
[5] G. Taroncher-Oldenburg, A. Marshall, *Nat. Biotechnol.* **2007**, *25*, 961.
[6] S. M. Elbashir, W. Lendeckel, T. Tuschl, *Genes Dev.* **2001**, *15*, 188–200.
[7] J. Liu, M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor, G. J. Hannon, *Science* **2004**, *305*, 1437–1441.
[8] J. J. Song, S. K. Smith, G. J. Hannon, L. Joshua-Tor, *Science* **2004**, *305*, 1434–1437.
[9] T. Röhl, J. Kurreck, *J. Neurochem.* **2006**, *99*, 371–380.
[10] C. Matranga, Y. Tomari, C. Shin, D. P. Bartel, P. D. Zamore, *Cell* **2005**, *123*, 607–620.
[11] T. A. Rand, S. Petersen, F. Du, X. Wang, *Cell* **2005**, *123*, 621–629.
[12] I. J. MacRae, E. Ma, M. Zhou, C. V. Robinson, J. A. Doudna, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 512–517.
[13] M. J. Clemens, A. Elia, *J. Interferon Cytokine Res.* **1997**, *17*, 503–524.
[14] M. R. Player, P. F. Torrence, *Pharmacol. Ther.* **1998**, *78*, 55–113.
[15] L. Manche, S. R. Green, C. Schmedt, M. B. Mathews, *Mol. Cell. Biol.* **1992**, *12*, 5238–5248.
[16] E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **2001**, *409*, 363–366.
[17] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, *Nature* **2001**, *411*, 494–498.
[18] S. Weitzer, J. Martinez, *Nature* **2007**, *447*, 222–226.
[19] D. R. Corey, *Nat. Chem. Biol.* **2007**, *3*, 8–11.
[20] G. L. Sen, H. M. Blau, *Nat. Cell Biol.* **2005**, *7*, 633–636.
[21] J. R. Bertrand, M. Pottier, A. Vekris, P. Opolon, A. Maksimenko, C. Malvy, *Biochem. Biophys. Res. Commun.* **2002**, *296*, 1000–1004.
[22] A. Grunweller, E. Wyszko, B. Bieber, R. Jahnel, V. A. Erdmann, J. Kurreck, *Nucleic Acids Res.* **2003**, *31*, 3185–3193.

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[23] A. Watanabe, M. Arai, M. Yamazaki, N. Koitabashi, F. Wuytack, M. Kurabayashi, *J. Mol. Cell. Cardiol.* **2004**, *37*, 691 – 698.
[24] T. Christoph, A. Grunweller, J. Mika, M. K. Schafer, E. J. Wade, E. Weihe, V. A. Erdmann, R. Frank, C. Gillen, J. Kurreck, *Biochem. Biophys. Res. Commun.* **2006**, *350*, 238 – 243.
[25] T. S. Zimmermann, A. C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M. N. Fedoruk, J. Harborth, J. A. Heyes, L. B. Jeffs, M. John, A. D. Judge, K. Lam, K. McClintock, L. V. Nechev, L. R. Palmer, T. Racie, I. Rohl, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A. J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H. P. Vornlocher, I. MacLachlan, *Nature* **2006**, *441*, 111 – 114.
[26] M. Bühler, D. Moazed, *Nat. Struct. Mol. Biol.* **2007**, *14*, 1041 – 1048.
[27] Review: W. Filipowicz, S. N. Bhattacharyya, N. Sonenberg, *Nat. Rev. Genet.* **2008**, *9*, 102 – 114.
[28] M. L. Yeung, Y. Bennasser, K. T. Jeang, *Curr. Med. Chem.* **2007**, *14*, 191 – 197.
[29] B. Berkhout, J. Haasnoot, *FEBS Lett.* **2006**, *580*, 2896 – 2902.
[30] P. Jin, R. S. Alisch, S. T. Warren, *Nat. Cell Biol.* **2004**, *6*, 1048 – 1053.
[31] N. Bushati, S. M. Cohen, *Annu. Rev. Cell Dev. Biol.* **2007**, *23*, 175 – 205.
[32] A. A. Aravin, G. J. Hannon, J. Brennecke, *Science* **2007**, *318*, 761 – 764.
[33] T. Watanabe, Y. Totoki, A. Toyoda, M. Kaneda, S. Kuramochi-Miyagawa, Y. Obata, H. Chiba, Y. Kohara, T. Kono, T. Nakano, M. A. Surani, Y. Sakaki, H. Sasaki, *Nature* **2008**, *453*, 539 – 543.
[34] O. H. Tam, A. A. Aravin, P. Stein, A. Girard, E. P. Murchison, S. Cheloufi, E. Hodges, M. Anger, R. Sachidanandam, R. M. Schultz, G. J. Hannon, *Nature* **2008**, *453*, 534 – 538.
[35] C. A. Stein, *Nat. Biotechnol.* **2001**, *19*, 737 – 738.
[36] T. Holen, M. Amarzguioui, M. T. Wiiger, E. Babaie, H. Prydz, *Nucleic Acids Res.* **2002**, *30*, 1757 – 1766.
[37] Review: J. Kurreck, *J. Biomed. Biotechnol.* **2006**, 83757.
[38] A. Khvorova, A. Reynolds, S. D. Jayasena, *Cell* **2003**, *115*, 209 – 216.
[39] D. S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin, P. D. Zamore, *Cell* **2003**, *115*, 199 – 208.
[40] Y. Tomari, C. Matranga, B. Haley, N. Martinez, P. D. Zamore, *Science* **2004**, *306*, 1377 – 1380.
[41] A. Reynolds, D. Leake, Q. Boese, S. Scaringe, W. S. Marshall, A. Khvorova, *Nat. Biotechnol.* **2004**, *22*, 326 – 330.
[42] D. Huesken, J. Lange, C. Mickanin, J. Weiler, F. Asselbergs, J. Warner, B. Meloon, S. Engel, A. Rosenberg, D. Cohen, M. Labow, M. Reinhardt, F. Natt, J. Hall, *Nat. Biotechnol.* **2005**, *23*, 995 – 1001.
[43] J. Hong, N. Wei, A. Chalk, J. Wang, Y. Song, F. Yi, R. P. Qiao, E. L. Sonnhammer, C. Wahlestedt, Z. Liang, Q. Du, *Biochem. Biophys. Res. Commun.* **2008**, *368*, 703 – 708.
[44] V. Patzel, S. Rutz, I. Dietrich, C. Koberle, A. Scheffold, S. H. Kaufmann, *Nat. Biotechnol.* **2005**, *23*, 1440 – 1444.
[45] U. Krueger, T. Bergauer, B. Kaufmann, I. Wolter, S. Pilk, M. Heider-Fabian, S. Kirch, C. Artz-Oppitz, M. Isselhorst, J. Konrad, *Oligonucleotides* **2007**, *17*, 237 – 250.
[46] R. Kretschmer-Kazemi Far, G. Sczakiel, *Nucleic Acids Res.* **2003**, *31*, 4417 – 4424.
[47] M. Overhoff, M. Alken, R. K. Far, M. Lemaitre, B. Lebleu, G. Sczakiel, I. Robbins, *J. Mol. Biol.* **2005**, *348*, 871 – 881.
[48] S. Schubert, A. Grunweller, V. A. Erdmann, J. Kurreck, *J. Mol. Biol.* **2005**, *348*, 883 – 893.
[49] Y. Shao, C. Y. Chan, A. Maliyekkel, C. E. Lawrence, I. B. Roninson, Y. Ding, *RNA* **2007**, *13*, 1631 – 1640.
[50] E. M. Westerhout, B. Berkhout, *Nucleic Acids Res.* **2007**, *35*, 4322 – 4330.
[51] Y. Ding, C. Y. Chan, C. E. Lawrence, *Nucleic Acids Res.* **2004**, *32*, W135 – 141.
[52] D. H. Kim, M. A. Behlke, S. D. Rose, M. S. Chang, S. Choi, J. J. Rossi, *Nat. Biotechnol.* **2005**, *23*, 222 – 226.
[53] D. Siolas, C. Lerner, J. Burchard, W. Ge, P. S. Linsley, P. J. Paddison, G. J. Hannon, M. A. Cleary, *Nat. Biotechnol.* **2005**, *23*, 227 – 231.
[54] Review: F. Buchholz, R. Kittler, M. Slabicki, M. Theis, *Nat. Methods* **2006**, *3*, 696 – 700.
[55] D. R. Corey, *J. Clin. Invest.* **2007**, *117*, 3615 – 3622.
[56] H. Y. Zhang, Q. Du, C. Wahlestedt, Z. Liang, *Curr. Top. Med. Chem.* **2006**, *6*, 893 – 900.
[57] M. Manoharan, *Curr. Opin. Chem. Biol.* **2004**, *8*, 570 – 579.
[58] J. Martinez, A. Patkaniowska, H. Urlaub, R. Luhrmann, T. Tuschl, *Cell* **2002**, *110*, 563 – 574.
[59] A. Grunweller, C. Gillen, V. A. Erdmann, J. Kurreck, *Oligonucleotides* **2003**, *13*, 345 – 352.
[60] C. Lorenz, P. Hadwiger, M. John, H. P. Vornlocher, C. Unverzagt, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4975 – 4977.
[61] J. Harborth, S. M. Elbashir, K. Vandenburgh, H. Manninga, S. A. Scaringe, K. Weber, T. Tuschl, *Antisense Nucleic Acid Drug Dev.* **2003**, *13*, 83 – 105.
[62] D. A. Braasch, S. Jensen, Y. Liu, K. Kaur, K. Arar, M. A. White, D. R. Corey, *Biochemistry* **2003**, *42*, 7967 – 7975.
[63] Y. L. Chiu, T. M. Rana, *RNA* **2003**, *9*, 1034 – 1048.
[64] F. Czauderna, M. Fechtner, S. Dames, H. Aygun, A. Klippel, G. J. Pronk, K. Giese, J. Kaufmann, *Nucleic Acids Res.* **2003**, *31*, 2705 – 2716.
[65] A. Santel, M. Aleku, O. Keil, J. Endruschat, V. Esche, G. Fisch, S. Dames, K. Loffler, M. Fechtner, W. Arnold, K. Giese, A. Klippel, J. Kaufmann, *Gene Ther.* **2006**, *13*, 1222 – 1234.
[66] A. Grünweller, R. K. Hartmann, *BioDrugs* **2007**, *21*, 235 – 243.
[67] S. Kauppinen, B. Vester, J. Wengel, *Handb. Exp. Pharmacol.* **2006**, *173*, 405 – 422.
[68] H. Kaur, B. R. Babu, S. Maiti, *Chem. Rev.* **2007**, *107*, 4672 – 4697.
[69] J. Elmen, H. Thonberg, K. Ljungberg, M. Frieden, M. Westergaard, Y. Xu, B. Wahren, Z. Liang, H. Orum, T. Koch, C. Wahlestedt, *Nucleic Acids Res.* **2005**, *33*, 439 – 447.
[70] O. R. Mook, F. Baas, M. B. de Wissel, K. Fluiter, *Mol. Cancer Ther.* **2007**, *6*, 833 – 843.
[71] S. Schubert, D. Rothe, D. Werk, H. P. Grunert, H. Zeichhardt, V. A. Erdmann, J. Kurreck, *Antiviral Res.* **2007**, *73*, 197 – 205.
[72] J. B. Bramsen, M. B. Laursen, C. K. Damgaard, S. W. Lena, B. R. Babu, J. Wengel, J. Kjems, *Nucleic Acids Res.* **2007**, *35*, 5886 – 5897.
[73] D. V. Morrissey, K. Blanchard, L. Shaw, K. Jensen, J. A. Lockridge, B. Dickinson, J. A. McSwiggen, C. Vargeese, K. Bowman, C. S. Shaffer, B. A. Polisky, S. Zinnen, *Hepatology* **2005**, *41*, 1349 – 1356.
[74] Review: Y. Shi, *Trends Genet.* **2003**, *19*, 9 – 12.
[75] T. R. Brummelkamp, R. Bernards, R. Agami, *Science* **2002**, *296*, 550 – 553.
[76] M. A. Carmell, L. Zhang, D. S. Conklin, G. J. Hannon, T. A. Rosenquist, *Nat. Struct. Biol.* **2003**, *10*, 91 – 92.
[77] G. Tiscornia, O. Singer, M. Ikawa, I. M. Verma, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1844 – 1848.
[78] J. Seibler, B. Kuter-Luks, H. Kern, S. Streu, L. Plum, J. Mauer, R. Kuhn, J. C. Bruning, F. Schwenk, *Nucleic Acids Res.* **2005**, *33*, e67.
[79] T. Christoph, G. Bahrenberg, J. De Vry, W. Englberger, V. A. Erdmann, M. Frech, B. Kogel, T. Rohl, K. Schiene, W. Schroder, J. Seibler, J. Kurreck, *Mol. Cell. Neurosci.* **2008**, *37*, 579 – 589.
[80] P. Honore, C. T. Wismer, J. Mikusa, C. Z. Zhu, C. Zhong, D. M. Gauvin, A. Gomtsyan, R. El Kouhen, C. H. Lee, K. Marsh, J. P. Sullivan, C. R. Faltynek, M. F. Jarvis, *J. Pharmacol. Exp. Ther.* **2005**, *314*, 410 – 421.

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[81] H. Hasuwa, K. Kaseda, T. Einarsdottir, M. Okabe, *FEBS Lett.* **2002**, *532*, 227 – 230.
[82] M. C. Golding, C. R. Long, M. A. Carmell, G. J. Hannon, M. E. Westhusin, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 5285 – 5290.
[83] Y. Zeng, E. J. Wagner, B. R. Cullen, *Mol. Cell* **2002**, *9*, 1327 – 1333.
[84] D. Boden, O. Pusch, R. Silbermann, F. Lee, L. Tucker, B. Ramratnam, *Nucleic Acids Res.* **2004**, *32*, 1154 – 1158.
[85] K. H. Chung, C. C. Hart, S. Al-Bassam, A. Avery, J. Taylor, P. D. Patel, A. B. Vojtek, D. L. Turner, *Nucleic Acids Res.* **2006**, *34*, e53.
[86] M. K. Rao, M. F. Wilkinson, *Nat. Protoc.* **2006**, *1*, 1494 – 1501.
[87] S. Kappel, Y. Matthess, M. Kaufmann, K. Strebhardt, *Nat. Protoc.* **2007**, *2*, 3257 – 3269.
[88] S. Kappel, Y. Matthess, B. Zimmer, M. Kaufmann, K. Strebhardt, *Nucleic Acids Res.* **2006**, *34*, 4527 – 4536.
[89] J. Seibler, A. Kleinridders, B. Kuter-Luks, S. Niehaves, J. C. Bruning, F. Schwenk, *Nucleic Acids Res.* **2007**, *35*, e54.
[90] H. Fechner, J. Kurreck in *Therapeutic Oligonucleotides* (Ed.: J. Kurreck), Royal Society of Chemistry Publishing, Cambridge **2008**, pp. 267 – 295.
[91] L. Fritsch, L. A. Martinez, R. Sekhri, I. Naguibneva, M. Gerard, M. Vandromme, L. Schaeffer, A. Harel-Bellan, *EMBO Rep.* **2004**, *5*, 178 – 182.
[92] S. Schubert, H. P. Grunert, H. Zeichhardt, D. Werk, V. A. Erdmann, J. Kurreck, *J. Mol. Biol.* **2005**, *346*, 457 – 465.
[93] D. Semizarov, L. Frost, A. Sarthy, P. Kroeger, D. N. Halbert, S. W. Fesik, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6347 – 6352.
[94] A. L. Jackson, S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, P. S. Linsley, *Nat. Biotechnol.* **2003**, *21*, 635 – 637.
[95] Y. Fedorov, E. M. Anderson, A. Birmingham, A. Reynolds, J. Karpilow, K. Robinson, D. Leake, W. S. Marshall, A. Khvorova, *RNA* **2006**, *12*, 1188 – 1196.
[96] A. Birmingham, E. M. Anderson, A. Reynolds, D. Ilsley-Tyree, D. Leake, Y. Fedorov, S. Baskerville, E. Maksimova, K. Robinson, J. Karpilow, W. S. Marshall, A. Khvorova, *Nat. Methods* **2006**, *3*, 199 – 204.
[97] E. M. Anderson, A. Birmingham, S. Baskerville, A. Reynolds, E. Maksimova, D. Leake, Y. Fedorov, J. Karpilow, A. Khvorova, *RNA* **2008**, *14*, 853 – 861.
[98] A. L. Jackson, J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J. M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova, P. S. Linsley, *RNA* **2006**, *12*, 1197 – 1205.
[99] C. A. Sledz, M. Holko, M. J. de Veer, R. H. Silverman, B. R. Williams, *Nat. Cell Biol.* **2003**, *5*, 834 – 839.
[100] A. J. Bridge, S. Pebernard, A. Ducraux, A. L. Nicoulaz, R. Iggo, *Nat. Genet.* **2003**, *34*, 263 – 264.
[101] V. Hornung, M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres, G. Hartmann, *Nat. Med.* **2005**, *11*, 263 – 270.
[102] A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock, I. MacLachlan, *Nat. Biotechnol.* **2005**, *23*, 457 – 462.
[103] M. Schlee, V. Hornung, G. Hartmann, *Mol. Ther.* **2006**, *14*, 463 – 470.
[104] E. Uhlmann in *Therapeutic Oligonucleotides* (Ed.: J. Kurreck), Royal Society of Chemistry Publishing, Cambridge, **2008**, pp. 142 – 162.
[105] M. E. Kleinman, K. Yamada, A. Takeda, V. Chandrasekaran, M. Nozaki, J. Z. Baffi, R. J. Albuquerque, S. Yamasaki, M. Itaya, Y. Pan, B. Appukuttan, D. Gibbs, Z. Yang, K. Kariko, B. K. Ambati, T. A. Wilgus, L. A. DiPietro, E. Sakurai, K. Zhang, J. R. Smith, E. W. Taylor, J. Ambati, *Nature* **2008**, *452*, 591 – 597.
[106] S. Saxena, Z. O. Jonsson, A. Dutta, *J. Biol. Chem.* **2003**, *278*, 44312 – 44319.
[107] J. G. Doench, C. P. Petersen, P. A. Sharp, *Genes Dev.* **2003**, *17*, 438 – 442.
[108] D. Grimm, K. L. Streetz, C. L. Jopling, T. A. Storm, K. Pandey, C. R. Davis, P. Marion, F. Salazar, M. A. Kay, *Nature* **2006**, *441*, 537 – 541.
[109] M. John, R. Constien, A. Akinc, M. Goldberg, Y. A. Moon, M. Spranger, P. Hadwiger, J. Soutschek, H. P. Vornlocher, M. Manoharan, M. Stoffel, R. Langer, D. G. Anderson, J. D. Horton, V. Koteliansky, D. Bumcrot, *Nature* **2007**, *449*, 745 – 747.
[110] V. Bitko, A. Musiyenko, O. Shulyayeva, S. Barik, *Nat. Med.* **2005**, *11*, 50 – 55.
[111] A. P. McCaffrey, L. Meuse, T. T. Pham, D. S. Conklin, G. J. Hannon, M. A. Kay, *Nature* **2002**, *418*, 38 – 39.
[112] R. Juliano, M. R. Alam, V. Dixit, H. Kang, *Nucleic Acids Res.* **2008**, *36*, 4158 – 4171.
[113] D. V. Morrissey, J. A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C. S. Shaffer, L. B. Jeffs, A. Judge, I. MacLachlan, B. Polisky, *Nat. Biotechnol.* **2005**, *23*, 1002 – 1007.
[114] Q. Ge, L. Filip, A. Bai, T. Nguyen, H. N. Eisen, J. Chen, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 8676 – 8681.
[115] Z. Medarova, W. Pham, C. Farrar, V. Petkova, A. Moore, *Nat. Med.* **2007**, *13*, 372 – 377.
[116] Z. Liu, M. Winters, M. Holodniy, H. Dai, *Angew. Chem.* **2007**, *119*, 2069 – 2073; *Angew. Chem. Int. Ed.* **2007**, *46*, 2023 – 2027.
[117] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H. P. Vornlocher, *Nature* **2004**, *432*, 173 – 178.
[118] J. J. Turner, S. Jones, M. M. Fabani, G. Ivanova, A. A. Arzumanov, M. J. Gait, *Blood Cells Mol. Dis.* **2007**, *38*, 1 – 7.
[119] M. Overhoff, G. Sczakiel, *EMBO Rep.* **2005**, *6*, 1176 – 1181.
[120] E. Song, P. Zhu, S. K. Lee, D. Chowdhury, S. Kussman, D. M. Dykxhoorn, Y. Feng, D. Palliser, D. B. Weiner, P. Shankar, W. A. Marasco, J. Lieberman, *Nat. Biotechnol.* **2005**, *23*, 709 – 717.
[121] T. C. Chu, K. Y. Twu, A. D. Ellington, M. Levy, *Nucleic Acids Res.* **2006**, *34*, e73.
[122] J. O. McNamara II, E. R. Andrechek, Y. Wang, K. D. Viles, R. E. Rempel, E. Gilboa, B. A. Sullenger, P. H. Giangrande, *Nat. Biotechnol.* **2006**, *24*, 1005 – 1015.
[123] P. Kumar, H. Wu, J. L. McBride, K. E. Jung, M. H. Kim, B. L. Davidson, S. K. Lee, P. Shankar, N. Manjunath, *Nature* **2007**, *448*, 39 – 43.
[124] K. Zhang, Q. Wang, Y. Xie, G. Mor, E. Sega, P. S. Low, Y. Huang, *RNA* **2008**, *14*, 577 – 583.
[125] S. Akhtar, I. F. Benter, *J. Clin. Invest.* **2007**, *117*, 3623 – 3632.
[126] C. Reinsch, E. Siepi, A. Dieckmann, S. Panzner in *Therapeutic Oligonucleotides* (Ed.: J. Kurreck), Royal Society of Chemistry Publishing, Cambridge, **2008**, pp. 226 – 240.
[127] I. MacLachlan in *Therapeutic Oligonucleotides* (Ed.: J. Kurreck), Royal Society of Chemistry Publishing, Cambridge, **2008**, pp. 241 – 266.
[128] M. L. Edelstein, M. R. Abedi, J. Wixon, *J. Gene Med.* **2007**, *9*, 833 – 842.
[129] D. Grimm, M. A. Kay, *Hematology Am. Soc. Hematol. Educ. Program* **2007**, 473 – 481.
[130] M. Cavazzana-Calvo, S. Hacein-Bey, G. de Saint Basile, F. Gross, E. Yvon, P. Nusbaum, F. Selz, C. Hue, S. Certain, J. L. Casanova, P. Bousso, F. L. Deist, A. Fischer, *Science* **2000**, *288*, 669 – 672.

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[131] S. Hacein-Bey-Abina, C. Von Kalle, M. Schmidt, M. P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C. S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J. I. Cohen, G. de Saint Basile, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Sigaux, J. Soulier, L. E. Leiva, M. Wissler, C. Prinz, T. H. Rabbitts, F. Le Deist, A. Fischer, M. Cavazzana-Calvo, *Science* **2003**, *302*, 415 – 419.
[132] T. Abbas-Terki, W. Blanco-Bose, N. Deglon, W. Pralong, P. Aebischer, *Hum. Gene Ther.* **2002**, *13*, 2197 – 2201.
[133] T. R. Brummelkamp, R. Bernards, R. Agami, *Cancer Cell* **2002**, *2*, 243 – 247.
[134] S. D. Henry, P. van der Wegen, H. J. Metselaar, H. W. Tilanus, B. J. Scholte, L. J. van der Laan, *Mol. Ther.* **2006**, *14*, 485 – 493.
[135] S. Lehrman, *Nature* **1999**, *401*, 517 – 518.
[136] R. Alba, A. Bosch, M. Chillon, *Gene Ther.* **2005**, *12 Suppl 1*, S18 – 27.
[137] W. Chen, M. Liu, Y. Jiao, W. Yan, X. Wei, J. Chen, L. Fei, Y. Liu, X. Zuo, F. Yang, Y. Lu, Z. Zheng, *J. Virol.* **2006**, *80*, 3559 – 3566.
[138] H. Fechner, L. Suckau, J. Kurreck, I. Sipo, X. Wang, S. Pinkert, S. Loschen, J. Rekittke, S. Weger, D. Dekkers, R. Vetter, V. A. Erdmann, H. P. Schultheiss, M. Paul, J. Lamers, W. Poller, *Gene Ther.* **2007**, *14*, 211 – 218.
[139] D. Grimm, M. A. Kay, *Curr. Gene Ther.* **2003**, *3*, 281 – 304.
[140] Z. Wang, H. I. Ma, J. Li, L. Sun, J. Zhang, X. Xiao, *Gene Ther.* **2003**, *10*, 2105 – 2111.
[141] J. D. Hommel, R. M. Sears, D. Georgescu, D. L. Simmons, R. J. DiLeone, *Nat. Med.* **2003**, *9*, 1539 – 1544.
[142] Z. Han, M. Gorbatyuk, J. Thomas, Jr., A. S. Lewin, A. Srivastava, P. W. Stacpoole, *Mitochondrion* **2007**, *7*, 253 – 259.
[143] C. Warnecke, Z. Zaborowska, J. Kurreck, V. A. Erdmann, U. Frei, M. Wiesener, K. U. Eckardt, *FASEB J.* **2004**, *18*, 1462 – 1464.
[144] M. Chatterjee-Kishore, C. P. Miller, *Drug Discovery Today* **2005**, *10*, 1559 – 1565.
[145] *Nat. Cell Biol.* **2003**, *5*, 489 – 490.
[146] T. R. Brummelkamp, S. M. Nijman, A. M. Dirac, R. Bernards, *Nature* **2003**, *424*, 797 – 801.
[147] H. M. Oosterkamp, H. Neering, S. M. Nijman, A. M. Dirac, W. J. Mooi, R. Bernards, T. R. Brummelkamp, *Br. J. Dermatol.* **2006**, *155*, 182 – 185.
[148] A. L. Brass, D. M. Dykxhoorn, Y. Benita, N. Yan, A. Engelman, R. J. Xavier, J. Lieberman, S. J. Elledge, *Science* **2008**, *319*, 921 – 926.
[149] M. R. Schlabach, J. Luo, N. L. Solimini, G. Hu, Q. Xu, M. Z. Li, Z. Zhao, A. Smogorzewska, M. E. Sowa, X. L. Ang, T. F. Westbrook, A. C. Liang, K. Chang, J. A. Hackett, J. W. Harper, G. J. Hannon, S. J. Elledge, *Science* **2008**, *319*, 620 – 624.
[150] N. Wajapeyee, R. W. Serra, X. Zhu, M. Mahalingam, M. R. Green, *Cell* **2008**, *132*, 363 – 374.
[151] S. T. Crooke, *Annu. Rev. Med.* **2004**, *55*, 61 – 95.
[152] S. Schubert, J. Kurreck, *Curr. Drug Targets* **2004**, *5*, 667 – 681.
[153] D. Haussecker, *Hum. Gene Ther.* **2008**, *19*, 451 – 462.
[154] J. Shen, R. Samul, R. L. Silva, H. Akiyama, H. Liu, Y. Saishin, S. F. Hackett, S. Zinnen, K. Kossen, K. Fosnaugh, C. Vargeese, A. Gomez, K. Bouhana, R. Aitchison, P. Pavco, P. A. Campochiaro, *Gene Ther.* **2006**, *13*, 225 – 234.
[155] V. Bitko, S. Barik, *BMC Microbiol.* **2001**, *1*, 34.
[156] Review: J. Haasnoot, E. M. Westerhout, B. Berkhout, *Nat. Biotechnol.* **2007**, *25*, 1435 – 1443.
[157] K. M. Brown, C. Y. Chu, T. M. Rana, *Nat. Struct. Mol. Biol.* **2005**, *12*, 469 – 470.
[158] L. Gitlin, J. K. Stone, R. Andino, *J. Virol.* **2005**, *79*, 1027 – 1035.
[159] D. Boden, O. Pusch, F. Lee, L. Tucker, B. Ramratnam, *J. Virol.* **2003**, *77*, 11531 – 11535.
[160] A. T. Das, T. R. Brummelkamp, E. M. Westerhout, M. Vink, M. Madiredjo, R. Bernards, B. Berkhout, *J. Virol.* **2004**, *78*, 2601 – 2605.
[161] K. J. von Eije, O. ter Brake, B. Berkhout, *J. Virol.* **2008**, *82*, 2895 – 2903.
[162] S. Merl, R. Wessely, *Oligonucleotides* **2007**, *17*, 44 – 53.
[163] H. S. Lee, J. Ahn, Y. Jee, I. S. Seo, E. J. Jeon, E. S. Jeon, C. H. Joo, Y. K. Kim, H. Lee, *J. Gen. Virol.* **2007**, *88*, 2003 – 2012.
[164] O. T. Brake, K. Hooft, Y. P. Liu, M. Centlivre, K. Jasmijn von Eije, B. Berkhout, *Mol. Ther.* **2008**, *16*, 557 – 564.
[165] J. Anderson, R. Akkina, *Gene Ther.* **2007**, *14*, 1287 – 1297.
[166] C. B. Coyne, J. M. Bergelson, *Cell* **2006**, *124*, 119 – 131.
[167] H. Fechner, S. Pinkert, X. Wang, I. Sipo, L. Suckau, J. Kurreck, A. Dorner, K. Sollerbrant, H. Zeichhardt, H. P. Grunert, R. Vetter, H. P. Schultheiss, W. Poller, *Gene Ther.* **2007**, *14*, 960 – 971.
[168] D. Werk, S. Schubert, V. Lindig, H. P. Grunert, H. Zeichhardt, V. A. Erdmann, J. Kurreck, *Biol. Chem.* **2005**, *386*, 857 – 863.
[169] M. J. Li, J. Kim, S. Li, J. Zaia, J. K. Yee, J. Anderson, R. Akkina, J. J. Rossi, *Mol. Ther.* **2005**, *12*, 900 – 909.
[170] L. Scherer, M. S. Weinberg, J. J. Rossiin *Therapeutic Oligonucleotides* (Ed.: J. Kurreck), Royal Society of Chemistry Publishing, Cambridge, **2008**, pp. 316 – 328.
[171] J. Devincenzo, J. E. Cehelsky, R. Alvarez, S. Elbashir, J. Harborth, I. Toudjarska, L. Nechev, V. Murugaiah, A. V. Vliet, A. K. Vaishnaw, R. Meyers, *Antiviral Res.* **2008**, *77*, 225 – 231.
[172] S. I. Pai, Y. Y. Lin, B. Macaes, A. Meneshian, C. F. Hung, T. C. Wu, *Gene Ther.* **2006**, *13*, 464 – 477.
[173] H. Wu, W. N. Hait, J. M. Yang, *Cancer Res.* **2003**, *63*, 1515 – 1519.
[174] A. Santel, M. Aleku, O. Keil, J. Endruschat, V. Esche, B. Durieux, K. Loffler, M. Fechtner, T. Rohl, G. Fisch, S. Dames, W. Arnold, K. Giese, A. Klippel, J. Kaufmann, *Gene Ther.* **2006**, *13*, 1360 – 1370.
[175] Y. A. Zhang, J. Nemunaitis, S. K. Samuel, P. Chen, Y. Shen, A. W. Tong, *Cancer Res.* **2006**, *66*, 9736 – 9743.
[176] R. Zukiel, S. Nowak, E. Wyszko, K. Rolle, I. Gawronska, M. Z. Barciszewska, J. Barciszewski, *Cancer Biol. Ther.* **2006**, *5*, 1002 – 1007.
[177] D. H. Kim, J. J. Rossi, *Nat. Rev. Genet.* **2007**, *8*, 173 – 184.
[178] P. G. Komarov, E. A. Komarova, R. V. Kondratov, K. Christov-Tselkov, J. S. Coon, M. V. Chernov, A. V. Gudkov, *Science* **1999**, *285*, 1733 – 1737.
[179] F. J. Smith, R. P. Hickerson, J. M. Sayers, R. E. Reeves, C. H. Contag, D. Leake, R. L. Kaspar, W. H. McLean, *J. Invest. Dermatol.* **2008**, *128*, 50 – 58.
[180] J. Elmén, M. Lindow, S. Schutz, M. Lawrence, A. Petri, S. Obad, M. Lindholm, M. Hedtjarn, H. F. Hansen, U. Berger, S. Gullans, P. Kearney, P. Sarnow, E. M. Straarup, S. Kauppinen, *Nature* **2008**, *452*, 896 – 899.

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